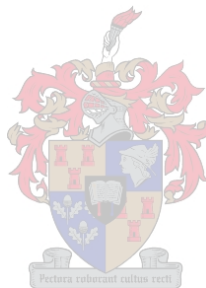


**ASSESSMENT OF INOCULATION TECHNIQUES TO EVALUATE APPLE  
RESISTANCE TO *PHYTOPHTHORA CACTORUM***

**Patience Thembelihle Zondo**



**Thesis presented in partial fulfillment of the requirements for the degree of Master of  
Science at the University of Stellenbosch**

**Supervisor: Sandra Denman  
Co-supervisor: Iwan Labuschagné  
March 2001**

## **Declaration**

I the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

## Summary

### **Assessment of inoculation techniques to evaluate apple resistance to *Phytophthora cactorum***

*Phytophthora cactorum* (Lebert & Cohn) Schröt. is the primary cause of crown, collar and root rot diseases of apple (*Malus domestica* Borkh.) trees worldwide. This pathogen is most destructive in commercial apple orchards under waterlogged soil conditions and has recently been identified as causing serious disease in some South African apple orchards. Crown, collar and root diseases are difficult to control because of their unpredictability and catastrophic nature. The use of resistant cultivars and rootstocks is economical and environmentally considerate. Therefore the need to develop screening techniques that will enable the selection of desirable disease resistant traits as part of an apple-breeding program in South Africa was identified. The work undertaken in this study was aimed at optimizing different techniques to test resistance.

Using two direct inoculation techniques (excised stem and intact stem) the aggressiveness of 10 isolates of *P. cactorum* on apple rootstocks was determined. The susceptibilities of five apple rootstocks were also compared. Results have shown isolate by rootstock interaction which means isolate aggressiveness was influenced by rootstocks tested. The selectivity of isolates suggests that there may be several strains of the pathogen. Population studies of the pathogen might contribute valuable information that could lead to better interpretation of results. Rootstock susceptibility was monitored *in vitro* throughout the season by inoculating at monthly intervals for 26-months. It was observed that during winter, rootstock susceptibility was low compared to high susceptibility during summer. These results have revealed new information regarding changes in the relative resistance of the different rootstocks over the growing season, e.g. the susceptibility pattern of rootstock MM106 occurred 1 to –2 months later than that of other rootstocks. This finding has important implications on the way in which resistance test results are interpreted, and emphasizes the importance of not relying on point sampling. Furthermore, useful information has been acquired regarding the epidemiology of the disease with regard to “windows of susceptibility”. The phenomenon of a phase shift in susceptibility of different rootstocks



needs to be tested on a broader scale to assess whether it has any practical application on resistance testing.

Although different inoculation techniques are applied in breeding programs, up to now there is no consensus on which technique works best for seedling selections. Since large numbers of individuals must be tested to improve the chances of detecting resistant genotypes, mass inoculations of young seedlings is a rapid way of identifying resistant individuals. Two different screening methods were tested during this study. Using the sand-bran technique, seedlings were transplanted onto inoculated soil and the root mass was used as a measure of resistance. In a second method zoospore inoculum was applied to seedlings growing in a sand:bark mixture at different concentrations and the seedlings were subjected either to water drenching or not. In both trials the aggressiveness of isolates differed significantly from each other and only higher inoculum concentrations were effective in causing disease. The age of seedlings used in tests emerged as an important factor. Seedlings under five-months-old should not be used. Drenching inoculated seedlings enhanced disease development but the production of sufficiently high numbers of zoospores was a laborious task. Thus, it is recommended that the sand-bran inoculum technique be tested with the drenching treatment for mass selection.

In conclusion this study confirms the importance of both choice of isolate and choice of inoculation intervals in determining susceptibility of rootstocks to infection. In spite of the fact that stem inoculation bioassays have limited resemblance to natural disease situations, these bioassays are useful for obtaining an indication as to whether genotypes have a degree of resistance and merit further testing. For this reason refinement of the stem inoculation bioassay is worthwhile pursuing. With regard to seedling trials, both the sand-bran and the zoospore technique appear promising but refinement of these techniques is necessary in order to present a more practical way of testing large volumes of seedlings.



## Opsomming

### **Evaluering van inokulasietegnieke om weerstand teen *Phytophthora cactorum* in appels te evalueer**

*Phytophthora cactorum* (Lebert & Cohn) Schröt. is die primêre oorsaak van kroon-, kraag en wortelvrot van appelbome (*Malus domestica* Borkh.). Dit is die mees verwoestende patogeen in kommersiële appelboorde waar daar versuipde toestande grond voorkom. *P. cactorum* is onlangs identifiseer as die patogeen wat ernstige kroon- en kraag-verotting in Suid Afrikaanse appelboorde veroorsaak. Kroon-, kraag- en wortelvrot is moeilik om te beheer as gevolg van die onvoorspelbaarheid en rampspoedige aard van die siekte. Die gebruik van kultivars en onderstamme wat weerstandbiedend is teen siektes en plaë is omgewingsvriendelik en is ekonomies van belang, dus het die behoefte ontstaan om inokulasietegnieke te ontwikkel om weerstandige saailinge te identifiseer en te selekteer as deel van 'n appelteelprogram in Suid Afrika. Die doelwit van hierdie studie is om verskillende inokulasietegnieke te toets en te verfyn om weerstand in appelsaailinge te identifiseer.

Deur gebruik te maak van twee inokulasietegnieke (die afgesnyde loot- en intakte loot tegniek), is die relatiewe aggressiwiteit van 10 isolate van *P. cactorum* en die vatbaarheid van vyf appelonderstamme ondersoek. Resultate het aangetoon dat die aggressiwiteit van die isolate gevarieer het na aanleiding van die onderstam wat getoets is. Die selektiwiteit van die isolate is 'n aanduiding dat daar moontlik verskeie rasse van die patogeen voorkom. Toekomstige studies op die populasiestruktuur van *P. cactorum* sal 'n belangrike bydrae maak tot die interpretasie van resultate oor weerstand en weerstandsteling.

Die vatbaarheid van onderstamme was ook in *in vitro* proewe ondersoek deur maandelikse inokulasies toe te pas oor 'n tydperk van 26 maande. Dit is opgemerk dat die onderstamvatbaarheid gedurende die winter laag was in vergelyking met die somer. Nie al die onderstamme het dieselfde gereageer gedurende verskillende toetstye nie. Hierdie resultate toon aan dat die relatiewe weerstand van verskillende onderstamme oor die groeiseisoen verskil, byvoorbeeld die vatbare reaksie van die onderstam 'MM106' het een tot twee maande later voorgekom in vergelyking met ander onderstamme wat getoets is. Hierdie bevinding het



belangrike implikasies op die interpretasie van weerstandstoetsing en beklemtoon die moontlike tekortkominge in enkelproefwaarnemings. Bruikbare inligting ten opsigte van die epidemiologie van die siekte is versamel wat beskryf kan word in terme van vensters van vatbaarheid wat verskil van onderstam tot onderstam. Verdere ondersoeke in die verband word aanbeveel.

Hoewel verskeie inokulasietegnieke bestaan om jong saailinge vir weerstand te toets, is daar tot op hierdie stadium nog nie ooreenstemming oor die beste tegniek wat toegepas moet word om saailingseleksie te doen nie. Omdat groot getalle saailinge getoets moet tydens die seleksieproses sal massa-inokulasie van saailinge die aangewese metode wees. Twee verskillende inokulasie tegnieke is getoets in die studie. Deur gebruik te maak van die sand-semel tegniek, is saailinge geplant in geïnfesteerde plantmedium, waartydens die wortelmassa van saailinge gebruik is om die reaksie op infeksie te kwantifiseer. Die soöspoor inokulasietegniek was toegepas op saailinge wat in 'n sand en basmengsel geplant is teen verskillende inokulumkonsentrasies. 'n Waterverdrenkingsbehandeling is ook getoets. In albei hierdie proewe het die aggressiwiteit van die isolate van mekaar verskil. Slegs die hoër inokulumkonsentrasies was effektief in die ontwikkeling van die siekte. Die ouderdom van saailinge is ook uitgewys as 'n belangrike faktor wat 'n rol speel in weerstandstoetsing. Saailinge jonger as 5 maande word nie aanbeveel vir hierdie toetse nie. Verdinking van saailinge het die voorkoms van die siekte verhoog, maar die produksie van groot getalle soöspore was 'n beperkende faktor in die uitvoering van die proef. Dit word aanbeveel dat die sand-semel inokulasietegniek verder evalueer moet word onder verskeie toestande, onder andere deur dit met verdrenkinghte kombineer.

Die belang van die keuse van isolaat en inokulasiedatum in bepaling van relatiewe weerstand van onderstamme teen *P. cactorum* is tydens die studie bevestig. Afgesien van die beperking van die staminokulasietegnieke in soverre dit verwyderd is van natuurlike infeksie, word die tegnieke aanbeveel om 'n indikasie te kry van die relatiewe weerstand van onderstamme. Beide die sand-semel en soöspoor tegnieke kan gebruik word om weerstandige saailinge te identifiseer, maar tegniese verfyning van hierdie tegnieke is nodig om saailinge in massa te evalueer.

*'Let us not become weary in doing good*

*For at the proper time*

*We will reap a harvest*

*If we do not give up. '*

Galatians 6:9



## **Acknowledgements**

**I would like to express my sincere gratitude and appreciation to the following people and institutions:**

The Agricultural Research Council for financial support;

Sandra Denman for your wisdom, insights and great assistance throughout this work;

Iwan Labuschagnè for initiating this study, your encouragement and valuable inputs regarding this work;

The ARC Infruitec-Nietvoorbij Breeding and Evaluation Team for technical assistance and encouragement;

The ARC Infruitec-Nietvoorbij Plant Biotechnology and Pathology Department for permission to use the facilities and for technical assistance;

Marita van der Rijst for professional statistical help;

Attie Louw of Redhill for supplying me with plant material throughout the study;

Celeste Linde for her initial involvement during the study; and

Family and friends for their constant support and encouragement.

To God be all Glory!!

## Contents

1. <i>Phytophthora cactorum</i> collar, crown and root rot of apple: an overview.	1
2. Evaluation of two direct inoculation techniques for the assessment of apple rootstock resistance against <i>Phytophthora cactorum</i> .	34
3. Use of an <i>in vitro</i> excised shoot bioassay to evaluate seasonal effects on apple rootstock resistance against <i>Phytophthora cactorum</i> .	55
4. Evaluation of two inoculation techniques for the assessment of resistance of apple seedlings against <i>Phytophthora cactorum</i> .	64

# **1. *Phytophthora cactorum* collar, crown and root rot of apple: An overview**

## **Introduction**

*Phytophthora cactorum* (Lebert & Cohn) Schröt. is the most destructive fungal disease in commercial apple orchards under waterlogged soils (Parker 1979; Harris 1991; Erwin and Ribeiro 1996). It is usually less of a problem in orchards with good drainage. Parker (1979) listed *Phytophthora* crown rot as one of the destructive diseases on commercially cultivated apple in most of the apple growing regions worldwide. In South Africa *P. cactorum* was first recorded as being pathogenic to apple (*Malus domestica* Borkh.) by Wijers (1937), who artificially inoculated apple fruits with an isolate recovered from antirrhinum by Mes (1934). Van der Merwe and Mathee (1973) were the first to attribute dieback of mature apple trees to *P. cactorum* in Western Cape orchards. Since then, in South Africa, *Phytophthora* crown and root rots have been considered important diseases of apple trees in waterlogged soil conditions where they cause decline in tree health and ultimately, tree death.

The Western Cape province is the major apple production region in South Africa. This is because the climate in combination with the cooler temperatures occurring at the slight altitudes in this region is suitable for deciduous fruit growth. Deciduous fruits grow best in zones with cool to cold, wet winters and warm, dry summers (Stander 1983; Anonymous 2000a). The total area under apple production in South Africa is 21 498 hectares (Anonymous 2000b). There are four major production areas in the Western Cape (Figure 1), namely, Elgin, Langkloof, Ceres, and Vyeboom which contribute 28%, 21%, 21% and 13% of the total hectares, respectively. Other apple producing areas within the Western Cape include Villiersdorp (9%), Montagu (2%) and Piketberg (2%). The rest of the 4% include the Free State production areas. Granny Smith is the most important cultivar contributing 33% of the total area planted to apples, followed by Golden Delicious (24%), Starking (10%) and Royal Gala (9%). The rest of the cultivars contribute 24%.





Figure 1 Major apple-producing areas in the Western Cape province.

Apples are the most economically important deciduous fruit in the Western Cape. In South Africa during 1997/1998 apple production amounted to \$59-million which was 38% of the total export value of deciduous fruits (Anonymous 2000c). In 1998/1999 season, 561 866 tons of apples were produced of which 25% went to direct sales in local markets, 33% was exported and 35% was processed, the remaining 7% made up various products such as dried fruits. The South African apple industry once reached R3-billion of exports, 80% of the exports went to Europe (Anonymous 1999). However, the world overproduction of apples, unusual weather patterns, disease outbreaks, demand of high quality fruit by consumers, competition from aggressive producers (New Zealand, Europe, China and Chile) all threaten to reduce the South African apple industry up to a third of its current size (Biseker 2000; Ludski 2000).

Phytophthora diseases of apple trees are difficult to control because of their unpredictability and catastrophic nature (Harris 1991). These diseases are usually controlled by the application of fungicides such as metalaxyl and fosetyl-Al, which often prove effective as a preventative measure, but are very expensive since multiple applications are necessary.



Furthermore, *Phytophthora cactorum* develop resistance to these fungicides if they are used continuously. With the emphasis on high standards of food safety, fungicide treated products are undesirable and alternative options to disease management must be sought. The use of cultivars and rootstocks that are resistant to pests and diseases is economical and environmentally considerate. The improvement of any plant species relies on the continuing development of new cultivars that meet changing market requirements. Therefore there is a need to develop screening techniques which will enable selection of desirable disease resistant traits as part of an apple-breeding program in South Africa. In a rootstock-breeding program it is necessary to have a reliable method by which rootstock resistance can be tested. A number of techniques have been used to test rootstock resistance in various apple-growing regions of the world (Borecki and Millikan 1969; Sewell and Wilson 1973; Bielenin 1977ab; Dakwa and Sewell 1981; Jeffers and Aldwinckle 1986; Utkhede and Quamme 1988; Browne and Mircetich 1993) but, there have been conflicting results and a lack of consensus on which techniques to employ in the testing process.

The aims of this study were to assess the applicability of a number of commonly recommended rootstock resistance screening techniques and to test a new and an existing seedling bioassay (pre-screening) in South Africa. The variation of aggressiveness of South African isolates of *P. cactorum* on apple rootstocks using the excised stem and the intact stem bioassays was assessed on commercial rootstocks of known resistance, that are commonly used in South Africa. Seasonal variation in the susceptibility of apple rootstocks to *P. cactorum* was monitored and two different seedling bioassays were tested so that a pre-screening technique could be optimized. As an initial step in these studies a review of the relevant literature was conducted and is presented hereunder.

### **Distribution and host range of *Phytophthora cactorum***

*Phytophthora cactorum* has been reported to be the primary cause of crown, collar and root rot diseases of apple trees in many parts of the world (Erwin *et al.* 1996). It is most commonly found in temperate regions and warm temperate areas, but limited in the tropics (Harris 1991). The countries in which it commonly occurs are listed by Erwin *et al.* (1996).



*Phytophthora cactorum* has been recorded as attacking more than 200 plant species in 150 genera representing 60 plant families (Erwin *et al.* 1996). It causes root, crown and collar rot, fruit rot, cankers, leaf blight, wilt and seedling blight. Although isolates of *P. cactorum* have been reported to be generally non-host specific (Erwin *et al.* 1996), there are reports of host preference expressed as differences in aggressiveness (Van der Scheer 1977; Hantula *et al.* 1997; Lilja *et al.* 1998). There have even been suggestions of cultivar specific strains (races) (McIntosh 1968; Aldwinckle *et al.* 1975). Although host specificity is rare, a single record of *P. cactorum* isolates from forest soil causing no infection on ginseng (Darmono *et al.* 1991) exists. Uncertainties about cross-pathogenicity, virulence and aggressiveness within and between populations of *P. cactorum* exist.

*Phytophthora cactorum* has been isolated from soil (Baines 1939; Smith 1955; McIntosh 1964; Sneh and McIntosh 1974; Gupta *et al.* 1985; Matheron *et al.* 1988). However, frequent failure to isolate the pathogen from soil led to ideas that the cause of declining apple trees could be attributable to weather conditions or unfavorable soil conditions (Anderson 1956). More recently, Horner and Wilcox (1996a) reported no activity of *P. cactorum* in orchard soils during early autumn until early spring due to low temperatures. The lack of detection of the pathogen in soil was attributed to its inactivity. Furthermore, Horner and Wilcox (1996b) defined three gradients in the distribution of *P. cactorum* in orchard soils. They reported that populations decreased from the bottom to the top of the slope. Populations also decreased with increasing distance from a tree trunk, and with increasing depth in the soil, thus the position of sampling may also have had an effect on its detection in soil.

*P. cactorum* has also been isolated from irrigation water, though the form in which it occurs and the significance of this inoculum in the development of crown rot disease is not yet known (McIntosh 1966; McIntosh 1972; Erwin *et al.* 1996). McIntosh (1964) reported to have isolated *P. cactorum* from irrigated soils only and not from non-irrigated soils.

### **The pathogen: *Phytophthora cactorum***

**Classification** The genus *Phytophthora* has long been included within the fungi. However, it is no longer classified in the kingdom Myceteae (the true fungi) on the basis of its evolutionary phylogeny, and it is now classified as a pseudofungi in the kingdom Chromista



(Agrios 1997). The kingdom Chromista contains brown algae, diatoms, oomycetes and similar organisms. *Phytophthora cactorum* is classified taxonomically as follows:

<b>Kingdom</b>	<i>Chromista</i>
<b>Phylum</b>	<i>Oomycota</i>
<b>Class</b>	<i>Oomycetes</i>
<b>Order</b>	<i>Peronosporales</i>
<b>Family</b>	<i>Pythiaceae</i>
<b>Genus</b>	<i>Phytophthora</i>
<b>Species</b>	<i>Phytophthora cactorum</i>

Oomycota are characterized by biflagellate zoospores with a long tinsel flagellum and a shorter whiplash flagellum. Sexual or resting spores (oospores) are also characteristic of the phylum Oomycota.

**Life cycle** *Phytophthora cactorum* is a homothallic fungus with a complex life cycle. The fungus consists of an asexual phase (Figure 2A), where motile zoospores arise from sporangia and a sexual phase (Figure 2B) resulting in oospore formation from the union of the antheridia with the oogonia. Chlamydospores are produced in unfavorable environmental conditions or fluctuating temperatures.

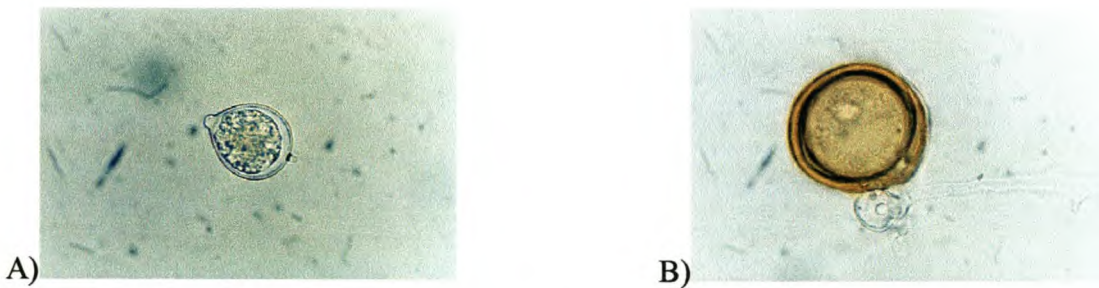


Figure 2 Reproduction structures of *Phytophthora cactorum*. A Papillate sporangium with a short pedicel B Oospore in oogonium with paragynous antheridium.

**Synonymy** The complete synonymy of *Phytophthora cactorum* (Lebert & Cohn) Schröt. 1886 according to Erwin *et al.* (1996) is as follows:

≡ *Peronospora cactorum* Lebert and Cohn (1870) (isolated from rotting cacti in Czechoslovakia)



- ≡ *Phloeophthora cactorum* (Lebert & Cohn) Wilson (1907)
- ≡ *Nozemia cactorum* (Lebert & Cohn) Pethybr. (1913) (isolated from rotting potato tubers)
- = *Peronospora sempervivi* Schenk (1875)
- = *Phytophthora fagi* (Hartig) Hartig (1876, 1880) (isolated from beech seedlings in Europe)
- = *Phytophthora omnivora* de Bary (1881) (reported on apples in Switzerland)
- = *Phytophthora pini* Leonian (1925) (isolated from the roots of *Pinus resinosa* in Minnesota)
- = *Phytophthora paeoniae* Cooper and Porter (1928) (described in Pennsylvania causing Peony blight).

### Morphological characteristics

*Phytophthora cactorum* was identified primarily on the markedly papillate sporangium, but more recently other papillate *Phytophthora* spp. have been identified (Hamm and Hansen 1983; Krober and Marwitz 1993; Kennedy and Duncan 1995). Other morphological features such as the size, shape and length-breadth ratio of sporangia, caducity and length of the pedicel are also important taxonomic criteria. The position of antheridium to the oogonium, the size and pleroticity of the oospores are important morphological characteristics of the oospores.

**Sporangia** They are distinctively papillate and caducous with short pedicels (less than 4 µm in length). They are readily produced on various agar media and sporangiophores are simple or in a loose sympodium with sporangia often clustered. They are normally borne terminally, occasionally intercalarly (Erwin *et al.* 1996). The inner sporangial wall becomes very thick and mucilaginous forming the papillum, the outer walls remain thin, firm and very resilient. Sporangia vary in size ranging from 45 x 33 µm to 28 x 20 µm depending on conditions under which they are produced (Blackwell 1943). The length-breadth ratio is  $1.2 \pm 0.1$  (Erwin *et al.* 1996) and the papillum ranges between 3 and 6 µm (Blackwell 1943).

Each sporangium has an average of thirty nuclei. A supply of cool fresh water to the sporangia causes the protoplasm to divide into uninucleate portions, which later become dense and round themselves off (Blackwell 1943). They then assume a pear shape and develop two lateral flagella. When papillae absorb water they burst through the thin outer pellicle and zoospores are released. After a period of motility (the duration of which depends on temperature, presence of osmotica, ionic composition of the soil water and proximity to plant



tissues), encystment occurs (Harris 1991). Cysts produce germ tubes that can grow up to 200  $\mu\text{m}$  in length before penetration occurs. If a sporangium has not produced zoospores it ripens into a conidium (Blackwell and Waterhouse 1931; Blackwell 1943).

**Conidia** They are mostly found in old cultures and in staling media (Waterhouse 1931; Blackwell 1943). Thus they might not be natural propagules encountered under field conditions. Chemical changes in both the basal plug and papilla render them less extensible and the protoplasm accumulates fatty reserves in a central globule and deposits a thin layer of cellulose on a wall. Once it has entered this stage the conidium is unable to produce zoospores and thus, has to germinate by a germ tube, which grows through the soft papillum and emerges apically (Blackwell 1943).

**Chlamydospores** They seldom occur in culture media (Erwin *et al.* 1996). Their production in culture is normally due to unfavorable environmental conditions or due to fluctuating temperatures. The majority of chlamydospores are produced terminally, rarely intercalarily (Blackwell 1943; Erwin *et al.* 1996). They are globose, have a double-layered wall (thickness of 1 to 1.5  $\mu\text{m}$ ) and the protoplasm contains oil reserves. Chlamydospores are developed at the tips of hyphal walls, and unlike oospores, they remain attached to these walls. Chlamydospore diameters ranges from 25 to 40  $\mu\text{m}$  (Domsch *et al.* 1980; Jeffers and Aldwinckle 1988; Jeffers and Wilcox 1990; Harris 1991; Erwin *et al.* 1996; Jee *et al.* 1997).

**Oogonia and antheridia** Oogonia and antheridia are the female and male sex organs, respectively. They initiate on adjoining lateral branches of the mycelium (Blackwell 1943). They are first observed when the hyphal tips of opposite mating strains come into close proximity and expand into two equal-sized, multinucleate spherical heads. One hyphal tip becomes larger with more nuclei and is recognized as the oogonial initial. The antheridial initial shows little enlargement and remains lying at the neck of the swelling oogonium and is described as paragynous. Blackwell (1943) measured the diameter of the oogonia to vary between 25 to 40  $\mu\text{m}$  and that of the antheridium from 12 to 15  $\mu\text{m}$ .

**The oospore** It is a major survival structure of *P. cactorum* in soil (Gisi 1983; Harris 1991; Horner *et al.* 1996a). It is formed immediately after the entry of the antheridial nuclei into the ooplasm. Oospores are binucleate. The protoplasm and nuclei left in the antheridia



degenerate into an oily mass, and the antheridium remains fixed to the oogonium wall until the oospore germinates. All oospores require a maturation period where they appear 'dormant' prior to germination (Blackwell *et al.* 1931). This period begins when the oospore reaches its minimum diameter within the oogonial wall (Blackwell 1943). Oospores have been reported to be plerotic (Erwin *et al.* 1996) with an average wall thickness of 2  $\mu\text{m}$ , but Oudemans and Coffey (1991) described oospores as aplerotic. The exospore is thin, smooth and transparent, built of pectic substances, mostly resistant and almost impermeable (Blackwell 1943). The endospore is composed of cellulose, proteins and other reserves.

### **Growth characteristics in culture media**

*Phytophthora cactorum* can grow at temperatures ranging from 2°C to 31°C, though its optimum growth temperature is 25°C. The macroscopic appearance of the mycelium varies with the medium in which it is growing. Mycelia are coenocytic, slender and slightly branched in a watery medium, but short, thick and highly branched, even swollen and nodular in a highly concentrated and relatively dry medium. The wall at the tip of each young growing hypha is thin and permeable. This is where absorption of nutrients takes place as well as growth in length (Blackwell 1943). The hyphae vary in diameter from 2 to 14  $\mu$  (Blackwell 1943). Vegetative branching is always monopodial (Erwin *et al.* 1996).

### **Molecular characterisation**

Using isozymes Oudemans *et al.* (1991) found considerable uniformity within a worldwide collection of *P. cactorum* isolates. They recognized only two electrophoretic groups, one of which was comprised of only two isolates from strawberry crowns, both from upper New York State. They showed that isolates of *P. cactorum* isolated from diverse geographic locations and host plants shared a high degree of similarity. Using random amplified polymorphic polymerase chain reaction (RAPD – PCR) analysis, Lilja *et al.* (1998) concluded that *P. cactorum* isolates from silver birch had higher genetic variability compared to isolates from strawberry in their DNA analysis. This implied that isolates from strawberry were uniform compared to polymorphic isolates from silver birch. Analysis of *P. cactorum* isolates using randomly amplified microsatellites (RAMS) suggested that isolates from different host plants have unique host specificities, thus tend to form different pathotypes in a given



geographic area (Hantula *et al.* 1997). Even though *P. cactorum* isolates from different hosts are indistinguishable morphologically, the application of molecular techniques to populations is aiding the elucidation of differences within a species. The outcome of this area of research will have important consequences for selection of isolates for resistance screening purposes.

### **Storage of isolates**

Maintenance of *Phytophthora* isolates in culture is difficult because the resting spores do not form readily. Therefore these isolates have to be frequently subcultured. For long-term storage four methods have been used successfully, viz. mineral oil, hemp seed, low temperature and sterile distilled water.

**Mineral oil technique** Wernham (1946) described a storage method whereby 3 – 5-day-old *Phytophthora* spp. cultures grown on V-8 juice agar slants were completely submerged in sterile mineral oil and stored at 10 – 15 °C for up to 2 years. The exception was with *Phytophthora infestans*, which had to be transferred every 9 – 12 months.

**Hemp seed technique** Small pieces of agar on which *Phytophthora* was growing were placed in screw cap bottles with sterile hemp seeds and distilled water (Raabe *et al.* 1973) than stored at room temperature (20-22°C). They reported that recovery of all *Phytophthora* spp. was possible after 1 – 4 years.

**Low temperature storage** *Phytophthora* cultures were cut into 8-mm discs using a cork borer and aseptically transferred into capped Pyrex tubes with sterile distilled water. The tubes were then stored in darkness at 5 °C (Marx and Daniel 1976). Cultures remained viable for 1 year.

**Sterile distilled water** *Phytophthora cactorum* isolates were stored at room temperature by transferring fresh cut blocks of culture into sterile distilled water in screw capped McCartney bottles (Boesewinkel 1976). Isolates could still be cultured after seven years.

### **Disease symptoms**

Crown rot of apples is a disease of the rootstock portion of the tree (Figure 3). Collar rot, on the other hand, is a disease of scion portion of the tree, affecting bark tissues of the lower



trunk at or above the soil line (Soteris and Mohamed 1985; Harris 1989; Jeffers *et al.* 1990). Crown rot was more prevalent in South Africa, but improved methods of irrigation and the use of more resistant rootstock reduced losses attributable to this disease. The first visible above ground signs of crown rot infection are purple-red colouring of the leaves or part of the leaf in late summer and autumn (Van der Merwe *et al.* 1973; Utkhede 1984a; Erwin *et al.* 1996). Cankers may extend from the point of origin into the root system where the pathogen girdles the roots and lower trunk resulting in stunted terminal growth and foliage discoloration (Jones and Sutton 1996). Van der Merwe *et al.* (1973) reported a definite margin between healthy and infected tissue showing different shades of brown. Later the tissue turns black and becomes soft and rotten.



Figure 3 Crown rot of apple trees caused by *Phytophthora cactorum*.

Bark lesions are of two types termed 'confined' or 'aggressive' (Dakwa *et al.* 1981). A confined lesion extends uniformly above and below the inoculation point. The lesion margin becomes clearly defined and surrounded by host callus tissue. An aggressive lesion is often irregular in its outline and is accompanied by a reddish-brown resin exudation. Aggressive lesion extends to the graft union where rootstock resistance checks it. The rate of lesion



development depends on the prevailing temperature, cultivar, scion/rootstock interaction, season and age of a shoot/root.

### **Factors affecting disease development**

**Climate** It influences the development of all diseases caused by *Phytophthora* spp. (Duniway 1983). Temperature and period of wetness are the most significant environmental factors influencing the infection of immature pear and apple trees by *P. cactorum* (Grove and Boal 1991). Disease severity increases with increasing temperature and period of wetness and different host types have different requirements for example, longer periods of wetness are required for infection of apples than for pears.

In tropical climates where temperatures are warm and the seasonal temperature changes are not severe *Phytophthora* species might be active throughout the year whereas in temperate climates some species may become inactive during cold months. Seasonal activities of host and environmental factors determine the epidemics caused by *Phytophthora* species. However inherent host resistance can moderate the effects of climate and the pathogen on disease development and more information on this is given in the section on resistance. It is important to note at this stage that resistance changes with physiological status of host (Bielenin 1977b).

**Temperature** Compared to the aerial environment the soil has relatively stable temperature, high and stable water status and very little solar radiation (Duniway 1983). The direct effects of physical factors on the pathogen (Harris 1991) largely determine epidemic development. However, the effects of physical factors on disease development can be influenced by a response of the host or by the activities of other organisms, which may stimulate or suppress the pathogen. Although the soil environment is less variable over time, some of these effects of physical factors affects the development of *Phytophthora* root rot in the soil, particularly temperature and wet soil conditions.

**Moisture** Saturated soil conditions are essential for the release and dispersal of zoospores in soil, but also predisposes plants such as alfalfa, to severe root rot (Utkhede and Smith 1996). Root rot incited by many *Phytophthora* spp. on a wide variety of plants is aggravated when the soil is saturated for prolonged periods (Duniway 1983). Water potential is the most significant



factor influencing sporangium production of soilborne *Phytophthora* species. Relative humidity approaching 100% is highly conducive to sporangial production. The susceptibility of bark to *P. cactorum* infection increased with increasing soil moisture from 61 to 96% (Harris 1991). The importance of water in the production and dispersal of zoospores explains why the epidemics of *Phytophthora* diseases of apples are linked with prolonged wet weather or deluges of rain. Moderate soil moisture and temperatures of 10°C favor the survival of zoospore cysts in soil. Zoospore cysts perished rapidly in air-dried soil. The extreme flexibility of growth and survival characteristics of the various stages of the life cycle enable *Phytophthora* spp. to thrive under unfavorable weather conditions.

**Host physiology** Susceptibility of aboveground parts of apple trees to *Phytophthora* species fluctuates seasonally (Bielenin 1977a; Browne and Mircetich 1995; Horner *et al.* 1996a). The fluctuations result from the interaction between the environment and the pathogen. The epidemic years for collar rot are those in which the period of tissue susceptibility significantly overlapped with the period of activity of *P. cactorum* (Harris 1991). Although the main climatic effects of moisture and temperature have a significant effect on disease development, the different physiological states of the host in the different seasons must not be underestimated. The resistance of plants to infection by soilborne *Phytophthora* species changes with the physiological status of the host. Bielenin (1977a) concurred that the bark of scion cultivars was inherently more resistant to *P. cactorum* during dormancy than it was during active growth. He also showed that there was a peak of susceptibility in spring during bloom. Sewell *et al.* (1973) in their study of trees inoculated with *P. cactorum* showed that resistance decreased as buds swelled and burst. They also found that resistance was lowest when leaf laminae were expanding, but increased sharply after shoot growth had commenced. Physiological changes associated with the transition from vegetative to a reproductive state may also affect the susceptibility of the rootstocks to disease (Jeffers *et al.* 1990). Thus the state of host physiology must be taken into account when undertaking resistance testing.

**Soil chemistry** Moderate to high balanced fertility has been reported to increase the severity of a number of diseases caused by soilborne *Phytophthora* species (Schmitthenner and Canaday 1983). High fertility effects may be due to high amounts of nitrogen in soil. Application of calcium nitrate, urea and sewage sludge increased the percentage of apple trees affected by *P. cactorum* (Utkhede and Smith 1995a). Application of nitrogen fertilizer



(ammonium nitrate) as a broadcast in spring, in spring and autumn, or by fertigation, caused higher levels of infection by *P. cactorum*. Application of nitrogen may be causing plant tissue to become soft and succulent, and this favors easy penetration of *P. cactorum* into the trunk and root tissues, which lead to infection (Utkhede *et al.* 1995a). It was reported that high pH results in increased severity of diseases caused by *Phytophthora* spp. (Gisi 1983) but, the growth rate of *P. cactorum* was little affected over the pH range 5.5 - 7.5, and greatly reduced at pH 4.5 (Jeffers *et al.* 1990).

**Inoculum sources** The concentration of inoculum in orchard soils is a critical factor in the epidemiology of the disease. Understanding the mechanisms of inoculum generation may lead to the development of more effective control measures. *Phytophthora* pathogens (*P. cactorum* predominantly) may be present in a site before apple trees are planted, or they may be introduced at the time of planting in infected root systems and infested medium adhering to roots. They may enter as contaminants of natural or irrigation water, or on feet of humans or animals, or on machinery (Harris 1991). Jeffers (1992) proved that unbudded apple rootstocks from propagation nurseries usually have a natural infestation with *Phytophthora* spp. (mainly *P. cactorum* and *P. cambivora*) and a major source of primary inoculum. The elimination or reduction of primary inoculum is one of the most important aspects of disease management. Moreover, Harris (1989) has shown that *P. cactorum* regenerates in non-host plants in orchard soils, thus weed control might be an important aspect of sanitation where weeds act as an alternate host.

## Control

*Phytophthora cactorum* diseases of apple trees are difficult to control because of their unpredictability and catastrophic nature (Harris 1991). An integrated approach to manage *Phytophthora* crown and root rot in the orchard is recommended.

## Cultural methods

**Nursery practices** The use of planting material free of *P. cactorum* is the most important means of disease avoidance in the establishment years of an orchard. In order to ensure disease free material, people that purchase rootstocks should insist on hot water treatments



(Nel 1983). Rootstocks should be propagated in pathogen free planting material. Regular inspection and testing would enable certification of material and this would reduce the incidence of pathogen spread from nurseries.

*Irrigation* Under wet, warm conditions, the fungus forms zoospores, which move by means of water in the soil and cause new infections. Poor field drainage and waterlogged soils present good conditions for successful infection. Adequate soil drainage and avoidance of sites with a high water table can reduce the Phytophthora collar, crown and root rot problem (Sharvelle 1979). Traditionally fruit trees have been irrigated by a surface method (basin, border/furrow irrigation), but most surface methods require high labor inputs (Feres and Gooldhamer 1990). The use of microsprinklers has become widespread in recent years for irrigation of deciduous fruit orchards. Major advantages of micro-sprinklers over conventional drip systems include less clogging, lower maintenance requirements, larger wetted surface areas from one emission point, better weed control due to less frequent applications and it is easier to identify the initial stages of emitter clogging. Sprinkler irrigation is becoming increasingly popular in countries with limited water supply. Water can be saved since with sprinkler irrigation, higher efficiencies are easier to obtain than with flood irrigation and in the Western Cape province, this is an increasingly important consideration (Shainberg and Oster 1978).

*Sanitation* Removal of sources of infection from which disease overwinters is essential (Cunningham 1925; Sharvelle 1979; Harris 1991). Destruction of infection sources does not eradicate disease, but reduces inoculum. Dead wood, weeds, weak shoots and wood with cankers in the cortex should be removed and burned during pruning. Cunningham (1925) advises that pruning operations should be completed before spring ploughing, so that any prunings left on the ground could be turned under during ploughing. Dead leaves should be disposed of by ploughing them under, or by burning them. Portions that were inaccessible to the tractor should be turned over with a spade. During picking, all infected fruit and fallen fruit should be removed first and buried away from the orchard (Harris 1991). Before unaffected fruits are picked, hands should be washed in hot water with carbolic soap (Cunningham 1925). Fruit skin injuries should be avoided, thus when picking, fruits should be placed on sterile containers.



**Soil conditions** Soil management can be of value as a method for disease control. By adjusting soil pH (so that it is unfavorable to the causal organism but favorable to the host) losses caused by *P. cactorum* can be reduced (Sharville 1979). Harris (1991) advises removal of soil from the tree base where the scion is at or below soil level.

**Chemical control** The difficulty in predicting disease outbreaks complicates the economic management of collar, crown and root rot with chemicals. *Phytophthora* associated diseases in apple orchards were traditionally controlled by the application of systemic fungicides such as metalaxyl and fosetyl-Al. These proved effective in most cases if used as preventative measures, but very expensive where multiple applications were necessary for long term control. Metalaxyl is usually applied as a drench around infected trees (Ellis *et al.* 1982; Utkhede 1984b; Ellis *et al.* 1986; Utkhede and Gupta 1988; Tidball and Linderman 1990; Utkhede and Smith 1991abc; Utkhede and Smith 1993). Resistance to metalaxyl has been reported in *P. cactorum* isolates tested *in vitro* (Utkhede *et al.* 1988). Therefore strict precautions should be taken when metalaxyl is used in the field. Field resistance can be delayed, either by keeping selection pressure low, by using a mixture of fungicides or by alternating fungicides with different modes of action.

Fosetyl-aluminium is a phloem-translocated systemic fungicide with both acropetal and basipetal movement (Long *et al.* 1989). The mechanism of action is not fully understood. It has been reported that it breaks down into phosphonic acid ( $H_3PO_3$ ) which protects rootstocks against *Phytophthora* diseases by stimulating host defense response (Utkhede and Smith 1995b). However, Fenn and Coffey (1984) demonstrated that an increase in antifungal compounds could be a secondary host response to a direct effect of phosphonic acid on the pathogen. Dercks and Buchenauer (1987) attributed the primary mode of action to a direct effect of fungicide on the parasite. It can be applied either as a drench (Utkhede 1987; Tidball *et al.* 1990; Utkhede *et al.* 1991bc), foliar spray (Utkhede *et al.* 1991c; Utkhede *et al.* 1993; Utkhede *et al.* 1995b; Ellis *et al.* 1988), stem canker paint (El-Hamalawi *et al.* 1995), or trunk injection (Long *et al.* 1989). No resistance to fosetyl-Al has been reported in *P. cactorum* isolates, but phytotoxicity has been reported when trees were injected during dormant periods (Long *et al.* 1989).



Dimethomorph is highly effective in the *in vitro* control of diseases caused by species of *Phytophthora*. The mode of action is unknown but it has been speculated that dimethomorph influences the molecular architecture of the wall (Kuhn *et al.* 1991) rather than carbohydrate metabolism or cell wall polymer production. No cross-resistance was reported between dimethomorph and phenylamide fungicides (e.g. metalaxyl). This means these fungicides can be used in alternation to avoid resistance developing.

Early diagnosis and timely curative treatment remain the most practical and economical ways of dealing with collar, crown and root rot in the absence of an efficient preventative method.

**Biological control** Janisiewicz and Covey (1983) demonstrated biological control by applying a siderophore-producing *Pseudomonas* sp. directly on the collar rot lesion caused by *P. cactorum* in the field. By removing decayed bark of fifteen-year-old trees and applying the bacterium directly onto the lesion, the spread of the lesion was arrested and a wound-healing callus formed. Cured trees remained alive and produced fruit.

A strain of *Enterobacter aerogenes* (Kruse) Hornaeche and Edwards, designated B8, was antagonistic to *P. cactorum* on corn meal agar (Utkhede and Gaunce 1983). This strain significantly reduced infection by three isolates of *P. cactorum* in sterile field soil (Utkhede *et al.* 1983) and also reduced the population of viable oospores in the top 30-mm soil (Gupta and Utkhede 1986). The mechanism of action involved was the production of an antifungal substance, characterized as neutral in pH and of low molecular weight, which inhibited the growth of the pathogen. At 21°C and pH 3.5, B8 produced maximum quantities of antifungal compound.

Utkhede *et al.* (1991a) tested twenty-one *Bacillus subtilis* (Ehrenb. Cohn) isolates and identified six that had the ability to control *Phytophthora* crown and root rot of MM106 apple rootstock in high density plantings. They reported a correlation between inhibition zones on corn meal agar and the percentage of seedlings infected under greenhouse tests. Utkhede (1984c) demonstrated that isolates of *B. subtilis* produced antibiotics inhibitory to mycelial growth of *P. cactorum* on corn meal agar. Gupta *et al.* (1986) discovered that the antifungal



compounds produced by *E. aerogenes* and *B. subtilis* inhibited the growth of *P. cactorum* and also reduced its potential to cause disease by reducing lesion lengths on dormant twigs.

*Trichoderma* spp. are well documented as effective biological control agents of plant diseases caused by soilborne fungi (Chet 1987), including some Pythiaceae fungi (Hadar *et al.* 1984). Research on *Trichoderma* spp. as control agents of root diseases of woody plants caused by *Phytophthora* spp. is limited. Greenhouse test results showed that *T. koningii* (Oudem.) and *T. harzianum* (Rifai) reduced crown and root rot of apples (Smith *et al.* 1990; Roiger and Jeffers 1991). Windham *et al.* (1986) attributed increased plant growth in the presence of *Trichoderma* spp. to the elimination of minor pathogens in the rhizosphere. For an effective biological control of crown rot, antagonists should multiply in the soil to produce the control effect in sufficient quantities to prevent growth of the pathogen. Biocontrol agents can be applied using pesticide sprayers (Utkhede 1987).

### **Disease resistance**

Apples have been selected for high productivity, good flavor, attractive appearance, and long storage life. In the process the innate resistance of species to many diseases and pests has been lost (Janick *et al.* 1996). The apple is grown as a composite tree consisting of a rootstock and a fruiting scion. Disease may attack both the rootstock and the scion cultivar, and having resistant rootstocks and resistant cultivars is essential. Rootstocks must have certain attributes such as ease of propagation either vegetatively or by seed, disease free stems, easy to bud, graft-compatible with all commercial scion cultivars and a root system that will provide adequate anchorage to support the tree without staking. Rootstocks should also be resistant to most pests and diseases, inducing early and heavy cropping.

Tree age is an important physical factor in the incidence of *Phytophthora* bark rots of apples. Trees more than four years of age are susceptible as the graft union can be in contact with soil (Miller and Pollard 1976; Soteris *et al.* 1985). Sewell *et al.* (1973) have attributed the absence of disease in young trees to 'juvenile' resistance and in trees of intermediate age, to disease escape. They also reported that apple tree resistance decreases with age and high resistance of young trees make them unsuitable for inoculation studies.



Brown (1975) concluded that there are two classes of resistance to *Phytophthora collar*, crown and root rot disease, polygenic and monogenic. McIntosh and Mellor (1954) reported that resistance in apple seedlings to zoospore inoculation with mixed isolates of *P. cactorum* was polygenic because of the wide variation from susceptible to very resistant among cultivars and progenies. Whereas Alston (1970) reported a monogenic dominant source of resistance (Pc) in 'Northern Spy' following wound inoculation of excised shoots. Three of 43 tested cultivars showed very high resistance and in 14 cultivars, resistance was better than in Northern Spy. This reaction appeared to be under simple genetic control. Watkins and Werts (1971) used zoospore inoculation on seedlings derived from crosses of rootstocks carrying the Pc gene and indicated that the presence of the resistance gene was not sufficient to explain the observed segregation. They also suggested that Pc may fail to confer resistance in plants with slow growing roots. It has been reported that Malling Merton rootstocks derive a high degree of resistance against collar rot disease from cultivar 'Northern Spy' (Cummins and Aldwinckle 1992).

Variations in techniques used, the inoculation of excised stems vs. intact plants and use of field observations vs. controlled studies complicates the evaluation of rootstock resistance. Rootstock MM106 showed an exceptionally high resistance when excised shoots were inoculated on the cambium with an agar disc containing *P. cactorum* mycelial growth (Sewell and Wilson 1959). Field observations (Sewell *et al.* 1973; Utkhede 1986; Utkhede *et al.* 1993) and *in vitro* inoculations (Barritt *et al.* 1990) confirmed MM106 as the most susceptible rootstock. In explaining the variability of MM106 in its reaction to *P. cactorum* infection Utkhede (1986) concluded that rootstock susceptibility varies from one region to another. The differential reaction of rootstocks may also be attributed to the levels of aggressiveness of *P. cactorum* isolates used in screening tests. Thus isolates of *P. cactorum* used for screening must carry genes that will enable it to attack a broad range of apple genotypes with the same level of aggressiveness. There is still a lack of understanding of the virulence and aggressiveness of *P. cactorum* isolates and this hampers the resistance testing procedure.

Even though excised, intact (Utkhede 1986; Browne *et al.* 1993) and *in vitro* (Jeffers *et al.* 1981) inoculations have resulted in infection and useful information, correlation with natural infection has not been satisfactory. These techniques involve wounding prior to



inoculation thus, only measure the susceptibility of the phloem-cambium region to the ingress of the pathogen, ignoring resistance to the penetration of the bark (Barritt *et al.* 1990). It has been suggested that seedlings that can not survive inoculation with *P. cactorum* will probably not be able to survive disease as mature trees (McIntosh 1968). Thus, seedling tests are conducted to eliminate very susceptible individuals at an early stage. This has been done by flooding seedlings to the soil line with suspensions of zoospores of aggressive *P. cactorum* isolates (McIntosh 1968; Watkins *et al.* 1971; Aldwinckle *et al.* 1975) and by transplanting seedlings into artificially infested soil (McIntosh 1965).

When breeding for resistance apple rootstock breeders should consider resistance not only to *P. cactorum* but also to other *Phytophthora* species that rootstocks may encounter (Browne *et al.* 1995). Most of the cultivated apples are diploid ( $2n = 34$ , Brown 1975). Any program of inbreeding by selfing is difficult to pursue because of high degree of self-incompatibility. Since the breeding of apples is based on selection from progenies produced by crossing extremely heterozygous parents, plant breeders must give consideration to parent selection (Brown 1975). Knowledge of the mode of inheritance may aid in the selection of parents, and in the choice of a breeding system.

### **Resistance screening in other industries**

There are several resistance screening methods used by different industries, other than the apple industry. These are discussed briefly below.

**The avocado industry** This industry has a few main methods employed in screening for resistance in rootstocks. Zentmyer and Mircetich (1960) reported a rapid and severe screening technique where seedlings were grown in tanks with complete nutrient solution. Once roots had developed *Phytophthora cinnamomi* Rands inoculum grown in potato dextrose agar for 7 days was placed in a cheesecloth bag and suspended in the nutrient solution in the tanks. After 10 days, percentage root rot was used as a measure of resistance. Prior to that a different method had been used by Zentmyer and Schroeder (1954). They transplanted seedlings into soil sampled around diseased trees, and rated percentage root rot as a measure of resistance. Zentmyer *et al.* (1962) reported good correlation of field data with greenhouse data. In the field they planted rootstock seedlings in soils artificially infested with *P. cinnamomi*. They



tested the occurrence of fungus both in soil and on roots. They concluded that the variety 'Duke' had more resistance than 'Topa Topa'. In yet another set of bioassays seedlings were rooted in vermiculite medium and inoculated with zoospores (Gabor and Coffey 1991). After 14 days percentage root rot was rated. Etiolated shoots were inoculated with a zoospore suspension and kept at 24 °C in the dark (Dolan and Coffey 1986; Gabor *et al.* 1991). Three days after incubation the development of *P. cinnamomi* was measured as lesion length. Electrolyte leakage of roots was determined by inoculating detached roots with extracts containing *P. cinnamomi* zoospores (Gabor *et al.* 1991). Conductivity of a bathing solution was measured after 4 hours of inoculation.

**The citrus industry** Afek *et al.* (1990) gave an overview of methods used to evaluate resistance of citrus rootstocks to *Phytophthora* species. In one method, root systems were immersed in concentrated zoospore suspension and transplanted in soil artificially infested with fungi. Percentage seedling survival was an estimate for resistance of parental genotypes used in crossing (Klotz *et al.* 1958; Furr and Carpenter 1961; Carpenter and Furr 1962). In a second method reported by Tsao and Garber (1960) and Grimm and Hutchison (1973), they planted inoculated seedlings in artificially infested soil and incubated them for 4 - 6 weeks at temperatures favorable to the pathogen. Root rot percentage was an estimate of susceptibility of the rootstock to the pathogen. Intact root systems of 2-3 month old seedlings were inoculated with a zoospore suspension and left to grow for 2-3 weeks. Percentage living roots was used to estimate resistance to the pathogen (Klotz and De Wolfe 1965; Cameron *et al.* 1972). In another method 1-year-old seedlings were inoculated in the stem bark with agar plugs containing *Phytophthora* mycelia. Symptom severity was used as a resistance estimate after 6-8 weeks (Klotz *et al.* 1965; Grimm *et al.* 1973; Whiteside and Knorr 1979). Afek *et al.* (1990) inoculated 3-month-old seedlings and kept them at high humidity (90-95%) and 24 °C for 4 days. Lesion length was measured and used as a criterion to measure resistance. Graham (1990) used chlamydospores as inoculum. Six-month-old seedlings were planted in soil artificially infested with chlamydospores. Number of rotted root tips were used as an indication of resistance of seedlings to disease. He found inoculum density to be in proportion with disease severity, i.e. the higher the inoculum density the more severe the symptoms of disease. Agostini *et al.* (1991) reported a fluctuation of propagule density of *P. parasitica* Dastur in the soil, its density differed depending on the site sampled. Stems of scions and

rootstocks were wounded and inoculated with agar containing chlamydospores. The mean percentage stem girdling and lesion area were taken as an estimate of resistance (Smith *et al.* 1991).

**The forestry industry** In the forestry industry pathogenicity tests are performed using 1 - 12-year-old trees by removal of bark using a cork borer. Agar plugs containing fungal mycelia replaces the bark and the wound is sealed (Boyce and Graves 1966; Davison and Tay 1983; Van der Westhuizen *et al.* 1993; Smith *et al.* 1994; Yuan and Mohammed 1999).

## Conclusions

Obviously, control of diseases of apples caused by *P. cactorum* are of major economic importance internationally and have recently been identified as such in South Africa. This could be because it has become crucial to keep costs of production and losses to a minimum so that South African products can compete in the international market. Resistance to *P. cactorum* is only one of the challenges facing the newly initiated apple rootstock breeding program in South Africa, but has high priority because of the difficulties involved and because losses in established orchards have serious economic implications. It is hoped that a contribution toward standardizing testing procedures will emerge with the work undertaken in this study.



## References

- Afek, U., Szejnberg, A. and Solel, Z. (1990) - A rapid method for evaluating citrus seedlings for resistance to foot rot caused by *Phytophthora citrophthora*. *Plant Disease* **74**: 66-68.
- Agostini, J.P., Timmer, L.W., Castle, W.S. and Mitchell, D.J. (1991) - Effect of citrus rootstocks on soil populations of *Phytophthora parasitica*. *Plant Disease* **75**: 296-300.
- Agrios, G.N. (1997) - Plant Pathology. Academic Press, United States of America. 4<sup>th</sup> edition.
- Aldwinckle, H.S., Polach, F.J., Molin, W.T. and Pearson, R.C. (1975) - Pathogenicity of *Phytophthora cactorum* isolates from New York apple trees and other sources. *Phytopathology* **65**: 989-994.
- Alston, F.H. (1970) - Resistance to collar rot, *Phytophthora cactorum* (Leb & Cohn) Schroet., in apple (abstract). *Report East Malling Research Station* pp. 143-145.
- Anonymous. (1999) - International tendencies in pome and stone fruit. *Deciduous Fruit Grower* **49**: 5-6.
- Anonymous. (2000a) - The Apple Industry. <http://www.capespan.co.za> (26/9/2000).
- Anonymous. (2000b) - The Economic Significance of the Agricultural Sector. <http://www.nda.agric.za> (26/9/2000).
- Anonymous. (2000c) - Abstracts of Agricultural Economics. National Department of Agriculture. Government Printer: Pretoria.
- Baines, R.C. (1939) - *Phytophthora* trunk canker or collar rot of apple trees. *Journal of Agricultural Research* **59**: 159-184.
- Barrit, B.H., Covey, R.P. and Dilley, M.A. (1990) - *In vitro* testing of the reaction of apple rootstocks to *Phytophthora cactorum*. *Fruit Varieties Journal* **44**: 23-25.
- Bielenin, A. (1977a) - Collar rot of apple trees. II . Seasonal fluctuations in susceptibility of apple trees to *Phytophthora cactorum* (Lebert & Cohn.) Schroeter. *Fruit Science Reports* **4**: 27-38.
- Bielenin, A. (1977b) - Collar rot of apple trees. I . Pathogenicity of isolates of *Phytophthora cactorum* (Lebert & Cohn.) Schroeter. *Fruit Science Reports* **4**: 15-26.

- Bisseker, C. (2000) - Cape apples no longer flavor of the month. *Financial Mail*: February 4: 46.
- Blackwell, E. (1943) - The life history of *Phytophthora cactorum* (Leb. and Cohn) Schroet. *Transactions of British Mycological Society* 26: 71-89.
- Blackwell, E.M. and Waterhouse, G.M. (1931) - Spores and spore germination in the Genus *Phytophthora*. *Transactions of British Mycological Society* 15: 294-321.
- Borecki, Z. and Millikan, D.F. (1969) - A rapid method for determining the pathogenicity and factors associated with pathogenicity of *Phytophthora cactorum*. *Phytopathology* 59: 245-247.
- Boyce, J.S. and Graves, A.A. (1966) - Monochaetia canker on Arizona Cypress in Georgia and South Carolina. *Plant Disease Reporter* 50: 482-483.
- Brown, A.G. (1975) - Apples. In: *Advances in Fruit Breeding* (Eds J. Janick and J.N. Moore), pp 3-37. Purdue University Press: Indiana.
- Browne, G.T. and Mircetich, S.M. (1993) - Relative resistance of thirteen apple rootstocks to three species of *Phytophthora*. *Phytopathology* 83: 744-749.
- Boosewinkel, H.J. (1976) - Storage of fungal cultures in water. *Transactions of the British Mycological Society* 66: 183-185.
- Cameron, J.W., Klotz, L.J., De Wolfe, T.A. and Soost, R.K. (1972) - Estimates of the resistance of *Citrus X Poncirus* hybrids to feeder root infection by *Phytophthora* spp. by a greenhouse seedling test. *Plant Disease Reporter* 56: 927-931.
- Carpenter, J.B. and Furr, J.R. (1962) - Evaluation of tolerance to root rot caused by *Phytophthora parasitica* in seedlings of Citrus and related genera. *Phytopathology* 52: 1277-1285.
- Chet, I. (1987) - *Trichoderma*-Application, mode of action, and potential as a biocontrol agent of soilborne plant pathogenic fungi. In: *Innovative Approaches to Plant Disease Control* (Ed I. Chet), pp. 137-159. John Wiley & Sons: New York
- Cooper, D.C. and Porter, C.L. (1928) - Phytophthora blight of peony. *Phytopathology* 18: 881-899.



- Cummins, J.N. and Aldwinckle, H.S. (1992) - Breeding apple rootstocks. In: *Plant Breeding Reviews* (Ed J. Janick) **9**: 295-394. John Wiley & Sons: New York.
- Cunningham, G.H. (1925) - Fungous diseases of fruit trees in New Zealand and their remedial treatment. The Brett Printing and Publishing Company Ltd: New Zealand. pp. 93-98.
- Dakwa, J.T. and Sewell, G.W.F. (1981) - Influence of rootstock type and time of inoculation on the resistance of five apple scion cultivars to collar rot caused by *Phytophthora cactorum*. *Journal of Horticultural Science* **56**: 357-362.
- Darmono, T.W., Owen, M.L. and Parke, J.L. (1991) - Isolation and pathogenicity of *Phytophthora cactorum* from forest and ginseng garden soils in Wisconsin. *Plant Disease* **75**: 610-612.
- Davidson, E.M. and Tay, F.C.S. (1983) - Twig, branch, and upper trunk cankers of *Eucalyptus marginata*. *Plant Disease* **67**: 1285-1287.
- de Bary, A. (1881) - Zur Kenntniss der Peronosporaeen (Information on peronosporas). *Bot. Zeit.* **39**: 585-625; 601-609; 617-625. (In German). (Cited in Erwin & Ribeiro 1996).
- Dercks, W. and Buchenauer, H. (1987) - Comparative studies on the mode of action of aluminium ethyl phosphite in four *Phytophthora* species. *Crop Protection* **6**: 82-89.
- Domsch, K.H., Gams, W. and Anderson, T. (1980) - *Phytophthora* de Bary 1876. Compendium of soil fungi. Academic Press: London.
- Dolan, T.E. and Coffey, M.D. (1986) - Laboratory screening technique for assessing resistance of four avocado rootstocks to *Phytophthora cinnamomi*. *Plant Disease* **70**: 115-118.
- Duniway, J.M. (1983) - Role of physical factors in the development of *Phytophthora* diseases. In: *Phytophthora: Its Biology, Taxonomy, Ecology, and Pathology* (Eds D.C. Erwin, S. Bartnicki-Garcia and P.H. Tsao), pp 175-187. The American Phytopathological Society: Minnesota.
- El-Hamalawi, Z.A., Menge, J.A. and Adams, C.J. (1995) - Methods of fosetyl-Al application and phosphonate levels in avocado tissue needed to control stem canker caused by *Phytophthora citricola*. *Plant Disease* **79**: 770-778.
- Ellis, M.A., Grove, G.G. and Ferree, D.C. (1982) - Effects of metalaxyl on *Phytophthora cactorum* and collar rot of apple. *Phytopathology* **72**: 1431-1434.



- Ellis, M.A., Ferree, D.C. and Madden, L.V. (1986) - Evaluation of metalaxyl and captafol soil drenches composted hardwood bark soil amendments, and graft union placement on control of apple collar rot. *Plant Disease* **70**: 24-26.
- Ellis, M.A., Wilcox, W.F. and Madden, L.V. (1988) - Efficacy of metalaxyl, fosetyl-aluminium, and straw mulch for control of strawberry leather rot caused by *Phytophthora cactorum*. *Plant Disease* **82**: 329-332.
- Erwin, D.C. and Ribeiro, O.K. (1996) - *Phytophthora* diseases worldwide. American Phytopathological Society Press: Minnesota.
- Fenn, M.E. and Coffey, M.D. (1984) - Studies on the *in vitro* and *in vivo* antifungal activity of fosetyl-Al and phosphorus acid. *Phytopathology* **74**: 606-611.
- Fereres, E. and Gooldhamer. (1990) - Irrigation of deciduous fruit and nut trees. In: *Irrigation of Agricultural Crops* (Eds B.A. Stewart and D.R. Nielsen), pp 987-1017.
- Furr, J.R. and Carpenter, J.B. (1961) - Program for breeding citrus rootstocks tolerant to *Phytophthora* root rot. *Proceedings of Florida State Horticultural Society* **74**: 18-23.
- Gabor, B.K. and Coffey, M.D. (1991) - Comparison of rapid methods for evaluating resistance to *Phytophthora cinnamomi* in avocado rootstocks. *Plant Disease* **75**: 118-120.
- Gisi, U. (1983) - Biophysical aspects of the development of *Phytophthora*. In: *Phytophthora: Its Biology, Taxonomy, Ecology, and Pathology* (Eds D.C. Erwin, S. Bartnicki-Garcia and P.H. Tsao), pp 109-119. American Phytopathological Society: Minnesota.
- Gisi, U., Binder, H. and Rimbach, E. (1985) - Synergistic interactions of fungicides with different modes of action. *Transactions of British Mycological Society* **85**: 299-306.
- Graham, J.H. (1990) - Evaluation of tolerance of citrus rootstocks to *Phytophthora* root rot in chlamydospore-infested soil. *Plant Disease* **74**: 743-746.
- Grimm, G.R. and Hutchison, D.J. (1973) - A procedure for evaluating resistance of citrus seedlings to *Phytophthora parasitica*. *Plant Disease Reporter* **57**: 669-672.
- Grove, G.G. and Boal, R.J. (1991) - Influence of temperature and wetness duration on infection of immature apple and pear fruit by *Phytophthora cactorum*. *Phytopathology* **81**: 1465-1471.



- Gupta, V.K., Rana, K.S. and Mir, N.M. (1985) - Variability in *Phytophthora cactorum* in India. In: *Ecology and Management of Soilborne Plant Pathogens* (Eds C.A. Parker, A.D. Rovira, K.J. Moore and P.T.W. Wong), pp. 169-171. American Phytopathological Society: Minnesota.
- Gupta, V.K. and Utkhede, R.S. (1986) - Factors affecting the production of antifungal compounds by *Enterobacter aerogenes* and *Bacillus subtilis*, antagonists of *Phytophthora cactorum*. *Journal of Phytopathology* **117**: 9-16.
- Hadar, Y., Harman, G.E. and Taylor, A.G. (1984) - Evaluation of *Trichoderma koningii* and *T. harzianum* from New York soils for biological control of seed rot caused by *Pythium* spp. *Phytopathology* **74**: 106-110.
- Hamm, P.B. and Hansen, E.M. (1983) - *Phytophthora pseudotsugae* a new species causing root rot of Douglas-fir. *Canadian Journal of Botany* **61**: 2626-2631.
- Hantula, J., Lilja, A and Parikka, P. (1997) - Genetic variation and host specificity of *Phytophthora cactorum* isolated in Europe. *Mycological Research* **101**: 565-572.
- Harris, D.C. (1989) - The significance of apple tree tissues for regeneration of *Phytophthora cactorum* in apple orchards. *Journal of Horticultural Science* **64**: 389-393.
- Harris, D.C. (1991) - The *Phytophthora* diseases of apple. *Journal of Horticultural Science* **66**: 513-544.
- Hartig, R. (1876) - Die Buchencotyledonen-Krankheiten (Cotyledon disease of beech). *Ztschr. Forst. Jdgdw.* **8**: 117-123. (In German). (Cited in Erwin & Ribeiro 1996).
- Hartig, R. (1880) - Der Buchenkeimlingspliz, *Phytophthora* (*Peronospora fagi* M.) (The beech seedling fungus, *Phytophthora* [*Peronospora fagi* M.]). *Untersuch. Forstb. Inst. Munchen.* **1**: 33-57. (In German). (Cited in Erwin & Ribeiro 1996).
- Horner, I.J. and Wilcox, W.F. (1996a) - Temporal changes in activity and dormant spore populations of *Phytophthora cactorum* in New York apple orchard soils. *Phytopathology* **86**: 1133-1139.
- Horner, I.J. and Wilcox, W.F. (1996b) - Spatial distribution of *Phytophthora cactorum* in New York apple orchard soils. *Phytopathology* **86**: 1122-1132.



- Janick, J, Cummins, J.N., Brown, S.K. and Hemmat, M. (1996) - Apples. In: *Fruit Breeding: Tree and Tropical Fruits* ( Eds J. Janick and J.N. Moore), pp 1-78. Wiley & Sons Inc: New York.
- Janisiewicz, W.J. and Covey, R.P. (1983) - Biological control of collar rot caused by *Phytophthora cactorum*. *Phytopathology* **72**: 533-538.
- Jee, H.J., Cho, W.D. and Kim, W.G. (1997) - *Phytophthora* diseases of apple in Korea. I. Occurrence of a destructive collar rot caused by *Phytophthora cactorum* (abstract). *Korean Journal of Plant Pathology* **13**: 139-144.
- Jeffers, S.N. (1992) - Preplant root treatments to reduce the incidence of *Phytophthora* species on dormant apple rootstocks. *Plant Disease* **76**: 12-19.
- Jeffers, S.N. and Aldwinckle, H.S. (1986) - Seasonal variation in extent of colonisation of two apple rootstocks by five species of *Phytophthora*. *Plant Disease* **70**: 941-945.
- Jeffers, S.N. Aldwinckle, H.S. (1988) - *Phytophthora* crown rot of apple trees: Sources of *Phytophthora cactorum* and *P. cambivora* as primary inoculum. *Phytopathology* **78**: 328-335.
- Jeffers, S.N., Aldwinckle, H.S., Burr, T.J. and Arneson, P.A. (1981) - Excised twig assay for the study of apple tree crown rot pathogens *in vitro*. *Plant Disease* **65**: 823-825.
- Jeffers, S.N. and Wilcox, W.F. (1990) - *Phytophthora* crown, collar and root rots. In: *Compendium of apple and pear diseases* (Eds A.L. Jones and H.S. Aldwinckle), pp. 43-45. The American Phytopathological Society: Minnesota.
- Jones, A.L. and Sutton, T.B. (1996) - Diseases of tree fruits in the East. Michigan State University: Michigan, pp. 46-47.
- Kennedy, D.M. and Duncan, J.M. (1995) - A papillate *Phytophthora* species with specificity to *Rubus*. *Mycological Research* **99**: 57-68.
- Klotz, L.J. and De Wolfe, T.A. (1965) - Tetrazolium, an indicator of extent of infection in *Phytophthora* root rot of citrus. *Plant Disease Reporter* **49**: 423-424.
- Klotz, L.J., De Wolfe, T.A. and Wong, P.P. (1958) - Decay of fibrous roots of citrus. *Phytopathology* **48**: 616-622.



- Krober, H. and Marwitz, R. (1993) - *Phytophthora tentaculata* sp. nov. und *Phytophthora cinnamomi* var. *parvispora* var. nov., zwei neue Pilz von Zierpflanzen in Deutschland (*Phytophthora tentaculata* sp. nov. und *Phytophthora cinnamomi* var. *parvispora* var. nov., two new fungi from ornamental plants in Germany). *Journal of Plant Disease Protection* **100**: 250-258.
- Kuhn, P.J., Pitt, D., Lee, S.A., Wakley, G. and Sheppard, A.N. (1991) - Effects of dimethomorph on the morphology and ultrastructure of *Phytophthora*. *Mycological Research* **95**: 333-340.
- Lebert, H. and Cohn, F. (1870) - Uber die Faule der Cactusstamme (On the rot of cactus stems). *Beitr. Biol. Pflanz.* **1**: 51-57. (In Germany). (Cited in Erwin & Ribeiro 1996).
- Leonian, L.H. (1925) - Physiological studies on the genus *Phytophthora*. *American Journal of Botany* **24**:444-548.
- Lilja, A., Karjalainen, R., Parikka, P., Kammiovirta, K. and Nuorteva, H. (1998) - Pathogenicity and genetic variation of *Phytophthora cactorum* from silver birch and strawberry. *European Journal of Plant Pathology* **104**: 529-535.
- Long, P.G., Miller, S.A. and Davis, L.K. (1989) - Duration of fungicidal effect following injection of apple trees with fosetyl-Al. *Journal of Phytopathology* **124**: 89-96.
- Ludski, H. (2000) - Bitter times fall on apple growers. *Sunday times Business Times*: August **27**: 7.
- Marx, D.H. and Daniel, W.J. (1976) - Maintaining cultures of ectomycorrhizal and plant pathogenic fungi in sterile water cold storage. *Canadian Journal of Microbiology* **22**: 338-341.
- Matheron, M.E., Young, D.J. and Matejka, J.C. (1988) - *Phytophthora* root and crown rot of apple trees in Arizona. *Plant Disease* **72**: 481-484.
- McIntosh, D.L. (1964) - *Phytophthora* spp. in soils of the Okanagan and Similkameen Valleys of British Columbia. *Canadian Journal of Botany* **42**: 1411-1415.
- McIntosh, D.L. (1965) - Studying the influence of soil temperatures on infection of fruit tree rootlets by *Phytophthora cactorum*. *Canadian Journal of Botany* **43**: 867-868.



- McIntosh, D.L. (1966) - The occurrence of *Phytophthora spp.* in irrigation systems in British Columbia. *Canadian Journal of Botany* **44**: 1591-1596.
- McIntosh, D.L. (1968) - Resistance to *Phytophthora cactorum* among open pollinated seedlings of apple varieties and species. *American Society for Horticultural Science* **93**: 71-76.
- McIntosh, D.L. (1972) - Effects of soil water suction, soil temperature, carbon and nitrogen ammendments, and host rootlets on survival in soil of zoospores of *Phytophthora cactorum*. *Canadian Journal of Botany* **50**: 269-272.
- McIntosh, D.L. and Mellor, F.L. (1954) - Crown rot of fruit trees in British Columbia, III Resistance trials on apple seedlings obtained from controlled crosses. *Canadian Journal of Agricultural Science* **34**: 539-541.
- Mes, M.G. (1934) - A wilt of Snapdragon (*Antirrhinum majus*) in South Africa. *South African Journal of Science* **31**: 281-287.
- Miller, P.R. and Pollard, H.L. (1976) - Multilingual compedium of plant diseases. The American Phytopathological Society: United States of America. pp 144.
- Nel, P.J. (1983) - Deciduous fruits and vines: Pests and Diseases and their Control. David Philip: Cape Town. pp 126-129.
- Oudemans, P. and Coffey, M.D. (1991) - Isozyme comparison within and among worldwide sources of three morphologically distinct species of *Phytophthora*. *Mycological Research* **95**: 19-30.
- Parker, K.G. (1979) - Common names of apple diseases. *Phytopathology News* **13**: 127-128. In: *Plant Breeding Reviews* **3**: 361-434 (Eds B.J. Parlman and G.A. White). AVI Publishing Company: United States of America.
- Pethybridge, G.H. (1913) - On the rotting of potato tubers by a new species of *Phytophthora* having a method of sexual reproduction hitherto undescribed. *Sci. Proc. Roy. Dublin Soc.* **13**: 529-565. (Cited in Erwin & Ribeiro 1996).
- Raabe, R.D., Hurliman, J.H. and Ermerson, R. (1973) - *Second International Plant Pathology Congress* (Abstract), Minnesota. (Cited in Ribeiro, 1978).



- Roiger, D.J. and Jeffers, S.N. (1991) - Evaluation of *Trichoderma spp.* for biological control of *Phytophthora* crown and root rot of apple seedlings. *Phytopathology* **81**: 910-917.
- Schenk, A. (1875) - Neue *Peronospora*: *P. sempevivi* (New *Peronospora*: *P. sempevivi*). *Bot.Zeit.* **33**: 690-693. (In German). (Cited in Erwin & Ribeiro 1996).
- Schmitthenner, A.F. and Canaday, C.H. (1983) - Role of chemical factors in development of *Phytophthora* diseases. In: *Phytophthora: Its Biology, Taxonomy, Ecology, and Pathology* (Eds D.C. Erwin, S. Bartnicki-Garcia and P.H. Tsao), pp 189-218. The American Phytopathological Society: Minnesota.
- Sewell, G.W.F. and Wilson, J.F. (1959) - Resistance trials of some apple rootstock varieties to *Phytophthora cactorum* (L. & C.) Schroet. *Journal of Horticultural Science* **34**: 51-58.
- Sewell, G.W.F. and Wilson, J.F. (1973) - *Phytophthora* rot of apple. Seasonal effects on infection and disease development. *Annals of Applied Biology* **74**: 149-158.
- Shainberg, I. and Oster, J.D. (1978) - Quality of irrigation water. International Irrigation information center. Bet Bagan: Israel. pp. 65.
- Sharvelle, E.G. (1979) - Plant disease control. AVI Publishing Company Inc.: Connecticut. pp. 16-34.
- Smith, H.C. (1955) - Collar-rot and crown-rot of apple trees. *The Orchardist of New Zealand* **28**: 16-21.
- Smith, G.S. Hutchison, D.J. and Henderson, C.T. (1991) - Comparative use of soil infested with chlamydospores to screen for relative susceptibility to *Phytophthora* foot rot in citrus cultivars. *Plant Disease* **75**: 402-405.
- Smith, H., Kemp, G.H.J. and Wingfield, M.J. (1994) - Canker and die-back of *Eucalyptus* in South Africa caused by *Botryosphaeria dothidea*. *Plant Pathology* **43**: 1031-1034.
- Smith, V.L., Wilcox, J.F. and Harman, G.E. (1990) - Potential for biological control of *Phytophthora* root and crown of apple by *Trichoderma* and *Gliocladium spp.* *Phytopathology* **80**: 880-885.
- Sneh, B. and McIntosh, D.L. (1974) - Studies on the behavior and survival of *Phytophthora cactorum* in soil. *Canadian Journal of Botany* **52**: 795-802.



- Soteros, J.J. and Mohamed, N.A. (1985) - Collar and crown rot of apples. *The Orchardist of New Zealand* **58**: 311-313.
- Stander, S. (1983) - Tree of life: The story of Cape Fruit. Saayam & Weber: Cape Town.
- Tidball, C.J. and Linderman, R.G. (1990) - Phytophthora root and stem rot of apple rootstocks from stool beds. *Plant Disease* **74**: 141-146.
- Tsao, P.H. and Garber, M.J. (1960) - Methods of soil infestation, watering and assessing the degree of root infection for greenhouse in situ ecological studies with citrus *Phytophthora*. *Plant Disease Reporter* **44**: 710-715.
- Utkhede, R.S. (1984a) - Effect of nitrogen fertilizers and wood composts on the incidence of apple crown rot in British Columbia. *Canadian Journal of Plant Pathology* **6**: 329-332.
- Utkhede, R.S. (1984b) - Effects of fungicides on apple crown rot caused by *Phytophthora cactorum*. *Pesticide Science* **15**: 241-246.
- Utkhede, R.S. (1984c) - Antagonism of isolates of *Bacillus subtilis* to *Phytophthora cactorum*. *Canadian Journal of Botany* **62**: 1032-1035.
- Utkhede, R.S. (1986) - *In vitro* screening of the world apple germplasm collection for resistance to *Phytophthora cactorum* crown rot. *Scientia Horticulturae* **29**: 205-210.
- Utkhede, R.S. (1987) - Chemical and biological control of crown rot of apple caused by *Phytophthora cactorum*. *Canadian Journal of Plant Pathology* **9**: 295-300.
- Utkhede, R.S. and Gaunce, A.P. (1983) - Inhibition of *Phytophthora cactorum* by a bacterial antagonist. *Canadian Journal of Botany* **61**: 3343-3348.
- Utkhede, R.S. and Gupta, V.K. (1988) - *In vitro* selection of strains of *Phytophthora cactorum* resistant to metalaxyl. *Journal of Phytopathology* **122**: 35-44.
- Utkhede, R.S. and Smith, E.M. (1991a) - Biological and chemical treatments for control of phytophthora crown and root rot caused by *Phytophthora cactorum* in a high density apple orchards. *Canadian Journal of Plant Pathology* **13**: 267-270.
- Utkhede, R.S. and Smith, E.M. (1991b) - *Phytophthora* and *Pythium* species associated with root rot of young apple trees and their control. *Soil Biology and Biochemistry* **23**: 1059-1063.



- Utkhede, R.S. and Smith, E.M. (1991c) - Effects of fosetyl-Al, metalaxyl, and *Enterobacter aerogenes* on crown and root rot of apple trees caused by *Phytophthora cactorum* in British Columbia. *Plant Disease* **75**: 406-409.
- Utkhede, R.S. and Smith, E.M. (1993) - Long-term effects of chemical and biological treatments on crown and root rot of apple trees caused by *Phytophthora cactorum*. *Soil Biology and Biochemistry* **25**: 383-386.
- Utkhede, R.S. and Smith, E.M. (1995a) - Effect of nitrogen form and application method on incidence and severity of *Phytophthora* crown and root rot of apple trees. *European Journal of Plant Pathology* **101**: 283-289.
- Utkhede, R. and Smith, E. (1995b) - Control of *Phytophthora* crown and root rot of apple trees with fosetyl-aluminium in new plantings. *Pesticide Science* **45**: 117-122.
- Utkhede, R.S. and Smith, E.M. (1996) - The effect of three irrigation practices on *Phytophthora* crown and root rot of apple trees under field conditions. *European Journal of Plant Pathology* **102**: 507-510.
- Utkhede, R.S. and Quamme, H.A. (1988) - Use of excised shoot assay to evaluate resistance to *Phytophthora cactorum* of apple rootstock cultivars. *Canadian Journal of Plant Science* **68**: 851-857.
- Van der Scheer, H.A.T. (1971) - Isolation of *Phytophthora cactorum* from soil in orchards and strawberry fields and differences in pathogenicity to apple. *Netherlands Journal of Plant Pathology* **75**: 65-72.
- Van der Merwe, J.J.H. and Matthee, F.N. (1973) - *Phytophthora* crown and root rot of apple trees in South Africa. *Phytophylactica* **5**: 55-58.
- Van der Westhuizen, I.P., Wingfield, M.J., Kemp, G.H.J. and Swart, W.J. (1993) - First report of the canker pathogen *Endothia gyrosa* on *Eucalyptus* in South Africa. *Plant Pathology* **42**: 661-663.
- Waterhouse, G.M. (1931) - The production of conidia in the genus *Phytophthora*. *Transactions of British Mycological Society* **15**: 311-321.
- Watkins, R. and Werts, J.M. (1971) - Pre-selection for *Phytophthora cactorum* (Leb. & Cohn) Schroet. resistance in apple seedlings. *Annals of Applied Biology* **67**: 153-156.

- Wernham, C.C. 1946. Mineral oil as a fungus culture preservative. *Mycologia* **38**: 691-692.
- Whiteside, J.O. and Knorr, I.C. (1979) - Susceptibility of different rough lemon collections to root rot, blight and *Alternaria* leaf spot. *Proceedings of Florida State Horticultural Society* **91**: 75-77.
- Wijers, E.E. (1937) - *Phytophthora* wilt in carnation plants. *South African Journal of Science* **34**: 194-213.
- Wilson, G.W. (1907) - Studies in North American Peronosporales. II . Phytophthoreae and Rhysottheceae. *Bull. Torrey Bot. Club* **34**:387-416. (Cited in Erwin & Ribeiro 1996).
- Windham, M.T., Elad, Y. and Baker, R. (1986) - A mechanism for increased plant growth induced by *Trichoderma* spp. *Phytopathology* **76**: 18-521.
- Yuan, Z.Q. and Mohammed, C. (1999) - Pathogenicity of fungi associated with stem cankers of eucalypts in Tasmania, Australia. *Plant Disease* **83**: 1063-1069.
- Zentmyer, G.A. and Mircetich, S.M. (1960) - Results with new method of testing for resistance to *Phytophthora* root rot of avocado. *California Avocado Society Yearbook* **44**: 107-109.
- Zentmyer, G.A. and Schroeder, C.A. (1954) - Tests of *Persea* species for resistance to *Phytophthora cinnamomi*. *California Avocado Society Yearbook* **38**: 163-164.
- Zentmyer, G.A., Thorn, W.A. and Burns, R.M. (1962) - Field trials for resistance to *Phytophthora* root rot. *California Avocado Society Yearbook* **40**: 169-173.



## 2. Evaluation of two inoculation techniques for the assessment of apple rootstock resistance against *Phytophthora cactorum*

### Abstract

Two existing direct inoculation techniques (excised stem and intact stem) were used to determine the aggressiveness of 10 isolates of *Phytophthora cactorum* on apple rootstocks. The susceptibilities of five apple rootstocks were also compared. Wounds were made with a cork borer on excised and intact stems that were inoculated with mycelial discs of the various isolates. Lesion length was used as the criterion by which aggressiveness of isolates as well as susceptibility of rootstocks was quantified. Results showed susceptibility of rootstocks varied according to isolate used. Therefore selection of isolates to determine the susceptibility of apple rootstocks to collar and crown rot is vital. Direct inoculations can only be used as an indication of levels of aggressiveness and susceptibility of rootstocks since they ignore the bark resistance thus only measure the susceptibility of the phloem - cambium region to infection.

### Introduction

The South African apple industry is almost entirely restricted to four production regions, all situated in the Western Cape province. These regions are Elgin, Langkloof, Ceres and Villiersdorp in order of importance (Anonymous 2000a). In South Africa apples are considered the most economically important deciduous fruit. In 1997/1998 production was valued at \$59 million which was 38% of the total value of deciduous fruits exported (Anonymous 2000b, Anonymous 2000c). Apple production in South Africa is expected to decrease due to worldwide overproduction of apples, unusual weather patterns and disease outbreaks (Bisseker 2000).

Collar and crown rot of apples caused by *Phytophthora cactorum* (Lebert & Cohn) Schröt., are insidious diseases which mostly only become evident on older trees established in orchards. Collar and crown rot of apple trees were reported for the first time in Western Cape



orchards in 1973 (Van Der Merwe and Mathee 1973). Since then, no reports of the disease have been published. However, in a recent survey it was estimated that in certain orchards as much as 20% of the orchard was lost to crown rot caused by *P. cactorum* (C. Linde, Institute of Plant Sciences/Phytopathology, Switzerland, personal communication). Apparently these diseases are also viewed as increasing problems on apple trees propagated on Malling (M) and Malling-Merton (MM) rootstocks in Canada (Utkhede and Smith 1993), and in other parts of the world (Jeffers and Aldwinckle 1986).

Outbreaks of crown rot occur at random and depend on excess soil water, suitable temperature and host susceptibility (Harris 1991). Crown rot is a disease of the rootstock in which the bark on the tree axis below soil level is invaded and destroyed, resulting in the death or severe debility of the tree. The disease is typified by the invasion of root-crown tissues of apple with the necrosis extending distally along the primary roots. Collar rot infection occurs on the scions. The fungus moves laterally and longitudinally and eventually girdles the tree by forming a necrotic collar around the trunk (Jeffers *et al.* 1982). Chemical control has been attempted in many areas but its effectiveness is limited. Little work has been done in the development of a biological control. Since the point of infection is the tree trunk, resistant rootstocks would be useful in reducing the infection rate thus reducing disease severity. In a rootstock-breeding program it is necessary to have a reliable method by which rootstock resistance can be tested. A number of techniques have been used to test rootstock resistance in various apple-growing regions of the world (Borecki and Millikan 1969; Sewell and Wilson 1973; Bielenin 1977b; Dakwa and Sewell 1981; Jeffers *et al.* 1986; Utkhede and Quamme 1988; Browne and Mircetich 1993). However, there have been conflicting reports about the best time of year (season) in which to carry out rootstock testing, and there have been reports of isolate - cultivar specificity (Aldwinckle *et al.* 1975).

The aim of this study was to assess the applicability of two existing direct inoculation techniques, the excised stem and the intact stem bioassays, using local isolates of *P. cactorum*, on commercial rootstocks of known resistance, that are commonly used in South Africa. The aggressiveness of the isolates was monitored as well as the relative resistance of the rootstocks.



## Methods

**Inoculum** Ten *P. cactorum* isolates obtained from four localities in the Western Cape province were used (Table 1). Isolations were made from roots and crowns of apple trees and strawberry plants. Symptomatic material was surface sterilized for 1 min in 1% sodium hypochlorite, rinsed for 1 min in distilled water and left to air dry in the laminar flow cabinet. Small segments of tissue from the margin between necrotic and healthy tissue were excised and placed on *Phytophthora* selective media (P<sub>5</sub>VP) (Jeffers and Martin 1986). Colonies that developed from the tissue were subcultured on V8 agar and identified using the key of Erwin and Ribeiro (1996). *Phytophthora cactorum* isolates were stored by in McCartney bottles with sterile distilled water at 25°C (Boesewinkel 1976). Two weeks prior to each inoculation date, isolates were cultured on potato dextrose agar (PDA, Difco) at 25°C.

**Excised stem inoculations** An excised shoot assay described by Browne *et al.* (1993) was used to test levels of aggressiveness of *P. cactorum* isolates on five apple rootstocks. Shoots of five different apple rootstocks, MM106 (susceptible, Jeffers *et al.* 1986), MM109 (moderately susceptible, Sewell and Wilson 1959), M7 (moderately resistant, Utkhede *et al.* 1993), M793 (moderately resistant, Sewell *et al.* 1959) and M25 (susceptible, Utkhede *et al.* 1988), were collected from a local nursery. Shoots were taken from the middle of one-year-old stems, and were cut to 20-cm lengths. Thirteen-millimeter bark discs were removed from each excised stem, using a cork borer to expose the xylem at two positions (5 cm away from each shoot end). The cut ends of the shoot were sealed at both ends with parafilm. The exposed xylem was inoculated by inserting a 13-mm diameter agar plug colonized with *P. cactorum* into the wound. Three shoots of each rootstock were inoculated with one isolate. Controls were inoculated with sterile PDA discs. Inoculated wounds were sealed with parafilm. Shoots were kept in the greenhouse at 80% relative humidity and a temperature of 25°C. After 8 days lesion lengths were measured on the bark. The excised stem trial was carried out in May (end of autumn), February (end of summer) and March (beginning of autumn) over three consecutive years (1998, 1999 and 2000). In 1998, tests were performed only in May.

**Statistical analysis** Mean lesion length for each isolate-rootstock combination were calculated. Data were analyzed for significant differences between year, season, isolates and



rootstocks, using Analyses of Variance (ANOVA), and Student's t-LSD tests were performed in the case of significant effects ( $p \leq 0.05$ ).

**Intact stem inoculations** Four rootstocks, MM109 (moderately susceptible, Sewell *et al.* 1959), M7 (moderately resistant, Utkhede *et al.* 1993), M25 (susceptible, Utkhede *et al.* 1988), MM111 (moderately susceptible, Utkhede *et al.* 1988), and one cultivar, Royal Gala (susceptible, personal observation) were used. Genotypes were grown in a greenhouse for one year before inoculations on intact stems were made following a similar procedure to that of excised stem inoculations. Except that intact stems were inoculated at one position (in the middle of the shoot) and the shoots were left intact on the rootstocks which were planted in plastic bags. Three plants were inoculated per isolate. Inoculated plants were arranged in a complete randomized block design in the greenhouse kept at 25°C. After 2 months lesion lengths were measured in the xylem tissue. The intact stem bioassay was repeated over two years (December 1998 and December 1999). Colonisation of the cambium in both trials was confirmed by re-isolation of the pathogen.

*Statistical analysis* The means of three lesion lengths per isolate-rootstock combination, were calculated. Levene's test for heterogeneity of variance (Snedecor and Cochran, 1980) was carried out on the data, and data were examined for a lack of normality using variance test for normality (Shapiro and Wilk, 1965). Significant heterogeneity and a lack of normality occurred. Data were transformed logarithmically, thereby reducing the heterogeneity of variance and lack of normality. Analysis of variance was carried out and Student's t-LSD tests were performed in the case of significant effects ( $p \leq 0.05$ ).

## Results

All isolates were pathogenic to apple rootstocks but significant interactions between the variables tested (namely, year, season, isolate and rootstock), occurred.

**Excised stem inoculations** Significant four way interaction (year x season x isolate x rootstock) prevented sensible interpretation of the results. Therefore, data for each year were analyzed separately.



There was no significant interaction between the isolates and the rootstocks in 1998 (Table 2), which allows interpretation of the main effects. There were highly significant differences between the rootstocks, and there were significant differences in aggressiveness among the isolates (Table 2). Essentially, there were three groups of isolates based on aggressiveness levels. These were a highly aggressive group of isolates (CP 706, CP 710, CP 713 and CP 716), the least aggressive isolates (CP 717, CP 725, CP 726 and CP 727) and a group of isolates that were intermediately aggressive (CP 728 and CP 729) (Figures 1 and 2). The range in lesion size was moderate (19 – 37 mm). There were also significant differences in susceptibility among rootstocks, none of the rootstocks were immune to disease. Rootstock MM106 was the most susceptible rootstock and MM109 and M7 were the least susceptible rootstocks. The rootstocks M25 and M793 were intermediately resistant (Figure 3).

Highly significant season x isolate x rootstock interaction occurred in 1999 (Table 2), thus, data for each season were analyzed separately (Table 3). There was no significant isolate x rootstock interaction in May (Table 3), thus main effects could be interpreted. No significant differences between rootstocks were detected. However, highly significant differences between isolates were evident (Table 3, Figure 4). Isolates CP 706, CP 710 and CP 713 were the most aggressive causing the largest lesions. The rest of the isolates were similar in aggressiveness levels, with isolates CP 725 and CP 727 being the least aggressive. This was the same as the previous year except that isolate CP 716 fell into the most aggressive group in 1998. In general, the overall aggressiveness of isolates was much reduced between 1998 and 1999, where in 1998 lesion lengths caused by aggressive isolates were in the order of 40 mm but in May 1999 they had dropped to about 20 mm. The range in lesion size in May 1999 was very small (16 – 22 mm).

In the seasons of February-99 and March-99, the isolate x rootstock interaction was highly significant (Tables 3). One of the main sources of interaction in February was rootstock M25, which developed much larger lesions when inoculated with CP 706 and CP 710 relative to the lesions developed with the other isolates (Figure 5). MM109 was highly susceptible when inoculated with isolates CP 710, CP 713 and CP 729. On the other hand, M793 appeared relatively resistant to isolates CP 710 and CP 713 (Figure 5). The smallest lesions were consistently formed on MM106, and M 7 was also moderately resistant (Figure



5). Isolates CP 716, CP 717 and CP 728 were least aggressive in this trial, which is fairly consistent with the previous trials. The range of difference in lesion size was large (8 – 114 mm).

In the March-99 trial the performance of rootstock MM106 was different to that in the February-99 trial. In February MM106 was relatively resistant, developing only small lesions with each isolate used (Figure 5). However, in March large lesions were formed consistently on this rootstock (Figure 6). The smallest lesions that developed on MM106 were those caused by isolates CP 710 and CP 727, where lesion lengths were 32 mm and 22 mm, respectively (Figure 6). M7 had small to moderate lesions in both February and March (Figures 5 and 6). Small lesions were formed consistently on MM109 in March, which is different from lesion development in February, where some of the biggest lesions were formed on this rootstock. The overall range in lesion lengths in March was between 16 – 85 mm.

In the year 2000 there was no significant season x isolate x rootstock interaction (Table 2). The only significant main effect was differences in aggressiveness of isolates (Table 2). Isolate CP 706 and CP 710 were the most aggressive isolates and the rest of the isolates were less aggressive (Figure 7). The range in lesion length was very small, 18 – 22 mm.

**Intact stem inoculations** Analysis of variance for the intact stem bioassay showed no significant year x isolate x rootstock interaction ( $P = 0.8900$ , Table 4). Therefore data collected over the two year period were pooled and means represent results of both trials (December 1998 and December 1999). It is notable that the year effect was highly significant ( $P = 0.0001$ , Table 4). In general, lesions were larger in 1998 than in 1999 (Figure 8).

Although the year x rootstock interaction ( $P = 0.0661$ , Table 4) was not significant at the 95 % confidence limit, it was significant at the 94 % confidence level and thus, merits note. In 1998, MM111 was relatively susceptible to the pathogen, whereas, in 1999, it was relatively resistant. In 1999 M25 appeared more susceptible than Royal Gala seedlings and M7. The other rootstocks showed a similar susceptibility response to infection in both trials (Figure 9). No significant interaction was observed between year and isolate ( $P = 0.8268$ ,



Table 4), which reinforces the relative consistency of isolate performance over the test period. However, the lesion lengths in 1998 were much larger than they were in 1999.

A highly significant rootstock x isolate interaction ( $P = 0.0001$ , Table 4) was obtained in this trial. One of the main sources of interaction occurred with isolates CP 706, CP 710 and CP 713 on rootstocks MM 109 and Royal Gala seedlings. Lesions on MM 109 were larger than lesions on MM 111 when isolates CP 706, CP 710 and CP 713 were used. The reverse occurred with the rest of the isolates (i.e. smaller lesions on MM 109 than on MM 111 [Figure 10]). Very big lesions were formed on Royal Gala seedlings when inoculated with isolates CP 706 and CP 713, but small lesions were formed with the rest of the isolates. No rootstocks were consistently resistant or consistently susceptible. Isolates CP 716, CP 717 and CP 726 appeared to have a low level of aggressiveness to all rootstocks. A large range in lesion length prevailed, 6 – 145 mm.

## Discussion

A great deal of variability in the behavior of isolates and rootstocks has been reported (Bielenin 1977ab; Browne *et al.* 1993; Harris 1991; Utkhede 1986) and was also apparent in the different seasons in this study. One problem that became evident with results, was the loss of aggressiveness of test isolates over the three-year period. Isolates should therefore be freshly isolated from infected material prior to use and should not be stored for long periods.

Seasonal variation in lesion development has been reported previously and results confirm this variation. However, it was found that not all the rootstocks responded in the same way at the different testing times. For example, MM 106 which is reportedly a susceptible rootstock (Jeffers *et al.* 1986) was moderately resistant in February, but became susceptible during March. Likewise, MM109 that is reported moderately susceptible (Sewell *et al.* 1959) was moderately susceptible in the February trials but became moderately resistant during March. The literature contains several contradictory reports on the susceptibility of apple cultivars and rootstocks in various seasons. Bielenin (1977a) did not observe differences among rootstocks during dormancy, rather the best separation of rootstock susceptibility occurred during bloom (spring to early summer). On the other hand, Utkhede *et al.* (1988) found the best separation of resistance properties when rootstocks were dormant.



In general it was found that during winter months when rootstocks were dormant, lesions were small and significant differences in susceptibilities of the various rootstocks was found only during first year of testing (1998). The lack of detection of differences among the rootstocks in the two later seasons (1999 and 2000) can be attributed to the loss of isolate aggressiveness over the three-year testing period. In May 1998 MM106 was the most susceptible rootstock, which is concordant with previous reports (Jeffers *et al.* 1986). However, MM109 reported moderately susceptible was the most resistant of all the rootstocks tested. Although rootstock susceptibility could be differentiated in 1998 trials, results were not all concordant with known susceptibilities. The range in mean lesion size in 1998 trials was very small (19 mm – 37 mm), and although differences are statistically significant, one may question the biological significance of these differences. Another factor that might affect results was the fact that there was no known resistant rootstock (for example M9) among rootstocks tested. The relative positioning of rootstocks to each other could change significantly if a resistant rootstock was included. It is therefore recommended that in all rootstock resistance testing standard susceptible and resistant rootstocks be included in the tests so that results could be comparable. Furthermore Browne and Mircetich (1993, 1996) have cautioned against placing undue emphasis on short-term results. The more knowledge is acquired about factors affecting the performance of rootstocks the better the testing procedures will become and the more reliable the results will be.

The significant interaction between rootstocks, isolates, and seasons has serious implications for rootstock testing. In tests over three winter seasons when the rootstocks were dormant there was no rootstock isolate interaction and differences in aggressiveness of isolates were evident. Generally, most of the isolates maintained their relative aggressiveness in this season over the years, although there was an overall loss of aggressiveness over time. Hence it appears that when rootstocks are dormant a good separation of highly aggressive and less aggressive isolates is possible. Utkhede *et al.* (1988) has also pointed this out. In trials when the rootstocks were actively growing in February and March, significant isolate rootstock interaction prevailed. Thus, the host plant largely influenced the relative aggressiveness of the isolates. This selectivity of isolates suggests that there may be several strains of the fungus (Bielenin 1977a). Molecular studies need to be undertaken to test this idea.



Intact stem bioassay tests were only conducted in December (mid-summer) over a two year period. As with the excised stem inoculation bioassay, there was significant isolate rootstock interaction. This emphasizes the need to elucidate the host pathogen interaction during the growing season. Furthermore, the fact that host isolate interaction does occur renders testing with a single isolate less reliable. Browne *et al.* (1993) have discussed this aspect. Population studies of the pathogen might contribute valuable information that could lead to better interpretation of results.

Although stem inoculation bioassays have limited resemblance to natural disease situations they are useful for obtaining an indication as to whether genotypes have a degree of resistance and merit further testing. These bioassays are easy to carry out, and are time-, space- and cost-efficient. However, the lack of natural infection process by using wounds to inoculate material limits the information obtained from these bioassays. These stem inoculation procedures only measure the susceptibility of the phloem and cambium tissues and ignore bark resistance. Furthermore, wounding may stimulate healing processes that could influence results. Jeffers *et al.* (1986) concluded that such inoculations did not measure resistance that would be expressed in the orchard. Therefore non-wound bioassays should be assessed.

These results emphasize the importance of choice of isolates in screening procedures, and the fact that more than one isolate should be used for more conclusive results when using direct inoculation methods. Also the need to ensure that isolates do not lose their aggressiveness in culture must be recognized. To date the lack of knowledge on host isolate interactions has impeded the reliability of such bioassays in the assessment of rootstock resistance to the crown and collar rot fungus, *P. cactorum*. It is therefore essential that further studies be undertaken to investigate the possibility of host/cultivar - isolate specificity. Environmental effects on disease epidemiology must also be taken into account. Since stem inoculation bioassays are convenient for testing rootstock resistance, pursuit of knowledge that will result in refinement of the technique should be supported.



## References

- Aldwinckle, H.S., Polach, F.J., Molin, W.T. and Pearson, R.C. (1975) - Pathogenicity of *Phytophthora cactorum* isolates from New York apple trees and other sources. *Phytopathology* **65**: 989-994.
- Anonymous. (2000a) - The Apple Industry. <http://www.capespan.co.za> (26/9/2000).
- Anonymous (2000b) - The Economic Significance of the Agricultural Sector. <http://www.nda.agric.za> (26/9/2000).
- Anonymous. (2000c) - Abstracts of Agricultural Economics. 2000. National Department of Agriculture. Government Printer: Pretoria.
- Bielenin, A. (1977a) - Collar rot of apple trees. I . Pathogenicity of isolates of *Phytophthora cactorum* (Leb. et Cohn.) Schroet. *Fruit Science Reporter* **4**: 15 - 26.
- Bielenin, A. (1977b) - Collar rot of apple trees. II . Seasonal fluctuations in susceptibility of apple trees to *Phytophthora cactorum* (Leb. et Cohn.) Schroet. *Fruit Science Reporter* **4**: 27-39.
- Bisseker, C. (2000) - Cape apples no longer flavor of the month. *Financial Mail*: February **4**:46.
- Boesewinkel, H.J. (1976) - Storage of fungal cultures in water. *Transactions of the British Mycological Society* **66**: 183-185.
- Borecki, Z. and Millikan, D.F. (1969) - A rapid method for determining the pathogenicity and factors associated with pathogenicity of *Phytophthora cactorum*. *Phytopathology* **59**: 245-247.
- Browne, G.T. and Mircetich, S.M. (1993) - Effects of month of inoculation on severity of disease caused by *Phytophthora* spp. in apple root crowns and excised shoots. *Phytopathology* **86**: 290-294.
- Browne, G.T. and Mircetich, S.M. (1996) - Effects of month of inoculation on severity of disease caused by *Phytophthora* spp. in apple root crowns and excised shoots. *Phytopathology* **86**: 290-295.



- Dakwa, J.T. and Sewell, G.W.F. (1981) - Influence of rootstock type and time of inoculation on the resistance of five apple scion cultivars to collar rot caused by *Phytophthora cactorum*. *Journal of Horticultural Science* **56**: 357-362.
- Erwin, D.C. and Ribeiro, O.K. (1996) - *Phytophthora* diseases worldwide. American Phytopathological Society Press: Minnesota. pp 96-141.
- Harris, D.C. (1991) - The *Phytophthora* diseases of apple. *Journal of Horticultural Science* **66**: 513-544.
- Jeffers, S.N. and Aldwinckle, H.S. (1986) - Seasonal variation in extent of colonization of two apple rootstocks by five species of *Phytophthora*. *Plant Disease* **70**: 941-945.
- Jeffers, S.N., Aldwinckle, H.S., Burr, T.J. and Arneson, P.A. (1982) - *Phytophthora* and *Pythium* species associated with crown rot in New York apple orchards. *Phytopathology* **72**: 533-538.
- Jeffers, S.N. and Martin, S.B. (1986) - Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease* **70**: 1038-1043.
- Sewell, G.W.F. and Wilson, J.F. (1959) - Resistance trials of some apple rootstock varieties to *Phytophthora cactorum* (L. & C.) Schroeter. *Journal of Horticultural Science* **34**: 51-58.
- Sewell, G.W.F. and Wilson, J.F. (1973) - *Phytophthora* rot of apple. Seasonal effects on infection and disease development. *Annals of Applied Biology* **74**: 149-158.
- Shapiro, S.S. and Wilk, M.B. (1965) - An analysis of variance test for normality (complete samples). *Biometrika* **52**: 591-611.
- Snedecor, G.W. and Cochran, W.G. (1980) - Statistical Methods. Seventh Edition. The Iowa State University Press. pp 253-254.
- Utkhede, R.S. (1986) - *In vitro* screening of the world germplasm collection for resistance to *Phytophthora cactorum* crown rot. *Scientia Horticulturae* **29**: 205-210.
- Utkhede, R.S. and Quamme, H.A. (1988) - The use of the excised shoot assay to evaluate resistance to *Phytophthora cactorum* of apple rootstock cultivars. *Canadian Journal of Plant Science* **68**: 851-857.



- Utkhede, R.S. and Smith, E.M. (1993) - Long-term effects of chemical and biological treatments on crown and root rot of apple trees caused by *Phytophthora cactorum*. *Soil Biology and Biochemistry* **25**: 383-386.
- Van Der Merwe, J.J.H. and Mathee, F.N. (1973) - *Phytophthora* crown and root rot of apple trees in South Africa. *Phytophylactica* **5**: 55-58.



**Table 1 Host and origin of isolates used in excised and intact stem bioassays**

Isolate name	Host of origin (Scion/Rootstock) <sup>A</sup>	Place of origin <sup>B</sup>
CP 706	Royal Gala/M793	Caledon
CP 710	Pink Lady/M25	Grabouw
CP 713	Pink Lady/MM106	Barrydale
CP 716	Pink Lady/MM106	Barrydale
CP 717	Pink Lady/MM106	Barrydale
CP 725	Selekta	Paarl
CP 726	Selekta	Paarl
CP 727	Selekta	Paarl
CP 728	Selekta	Paarl
CP 729	Selekta	Paarl

<sup>A</sup> - Seleкта is a strawberry plant.

<sup>B</sup> – All isolates used were from the Western Cape province.



**Table 2 Analysis of variance of apple rootstocks inoculated with ten isolates of *Phytophthora cactorum* using an excised stem inoculation technique over three years**

Source of variation	Degrees of freedom	F ratio	P value
<b>1998</b>			
Rep	2	0.02	0.9837
Isolate	9	14.15	0.0001
Rootstock	4	10.59	0.0001
Isolate x Rootstock	36	1.20	0.2435
<b>1999</b>			
Season	2	63.06	0.0001
Season(Rep)	6	1.29	0.2618
Isolate	9	15.93	0.0001
Rootstock	4	9.94	0.0001
Isolate x Rootstock	36	2.91	0.0001
Season x Isolate	18	3.49	0.0001
Season x Rootstock	8	15.98	0.0001
Season x Isolate x Rootstock	72	2.91	0.0001
<b>2000</b>			
Season	2	1.20	0.3035
Season(Rep)	6	0.84	0.5429
Isolate	9	2.32	0.0156
Rootstock	4	0.56	0.6909
Isolate x Rootstock	36	1.19	0.2157
Season x Isolate	18	1.26	0.2143
Season x Rootstock	8	0.86	0.5550
Season x Isolate x Rootstock	72	0.92	0.6658

**Table 3 Analysis of variance of apple rootstocks inoculated with ten isolates of *Phytophthora cactorum* using an excised stem inoculation technique in different seasons of 1999**

Source of variation	Degrees of freedom	F ratio	P value
<b>February</b>			
Rep	2	0.18	0.8326
Isolate	9	6.89	0.0001
Rootstock	4	13.36	0.0001
Isolate x Rootstock	36	3.20	0.0001
<b>March</b>			
Rep	2	2.32	0.1041
Isolate	9	8.12	0.0001
Rootstock	4	15.95	0.0001
Isolate x Rootstock	36	2.37	0.0004
<b>May</b>			
Rep	6	1.99	0.1417
Isolate	9	4.47	0.0001
Rootstock	4	1.22	0.3070
Isolate x Rootstock	36	1.25	0.1958



**Table 4 Analysis of variance of apple genotypes inoculated with ten isolates of *Phytophthora cactorum* using an intact stem bioassay over two years**

Source of variation	Degrees of freedom	F ratio	P value
Year	1	70.47	0.0001
Year (Rep)	4	0.95	0.4364
Isolate	9	9.34	0.0001
Rootstock	4	6.50	0.0001
Isolate x Rootstock	36	2.60	0.0001
Year x Isolate	9	0.56	0.8268
Year x Rootstock	4	2.24	0.0661
Year x Isolate x Rootstock	36	0.71	0.8900



Figure 1 Lesions caused by a less aggressive and a highly aggressive isolate of *Phytophthora cactorum* on MM106. From left to right: control, isolate CP 725 and isolate CP 713.

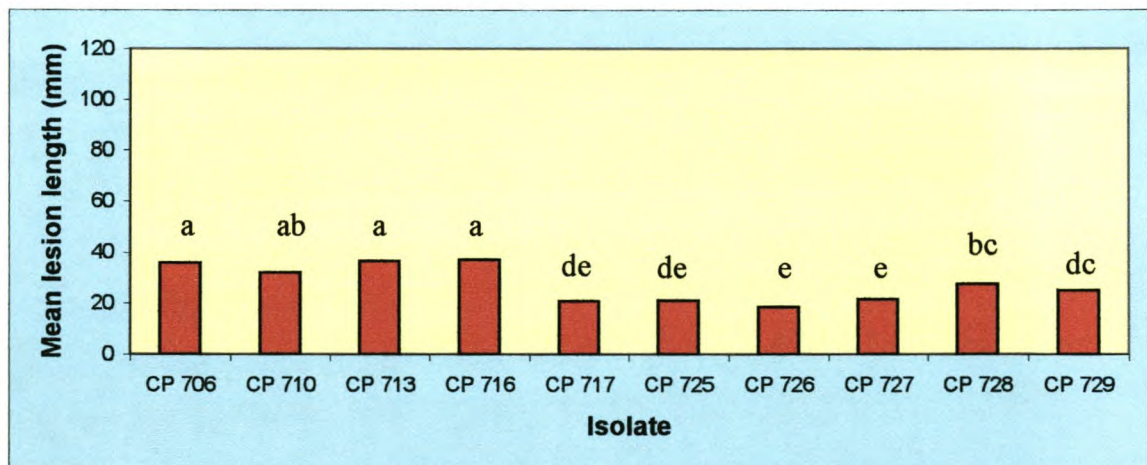


Figure 2 Aggressiveness of isolates expressed as mean lesion length for May 1998 using the excised stem technique. Means were calculated on the combined data of five rootstocks. Bars topped by the same letter do not differ significantly ( $P \leq 0.05$ ).



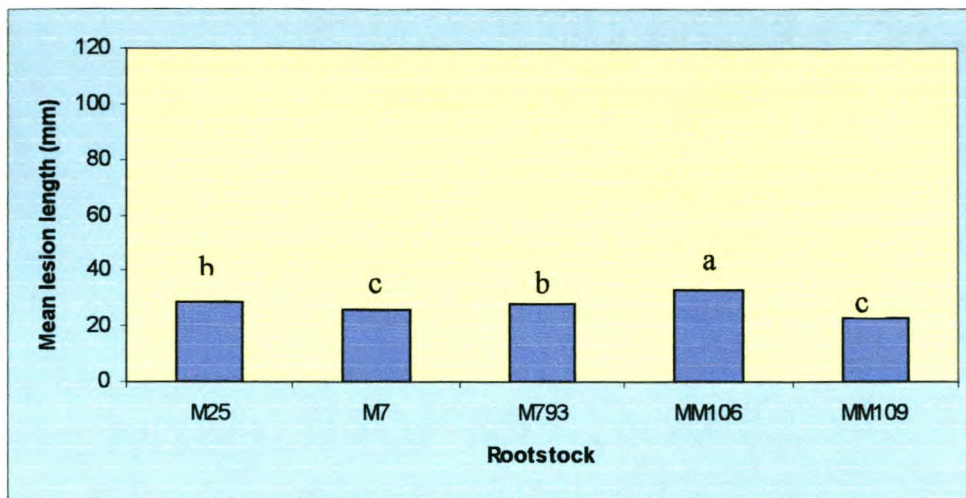


Figure 3 Rootstock susceptibility quantified as mean lesion length after inoculation in May 1998 using the excised stem bioassay. Means were calculated on the combined data of thirty isolates. Each bar represents means of 90 lesion lengths. Bars topped by the same letter do not differ significantly ( $P \leq 0.05$ ).

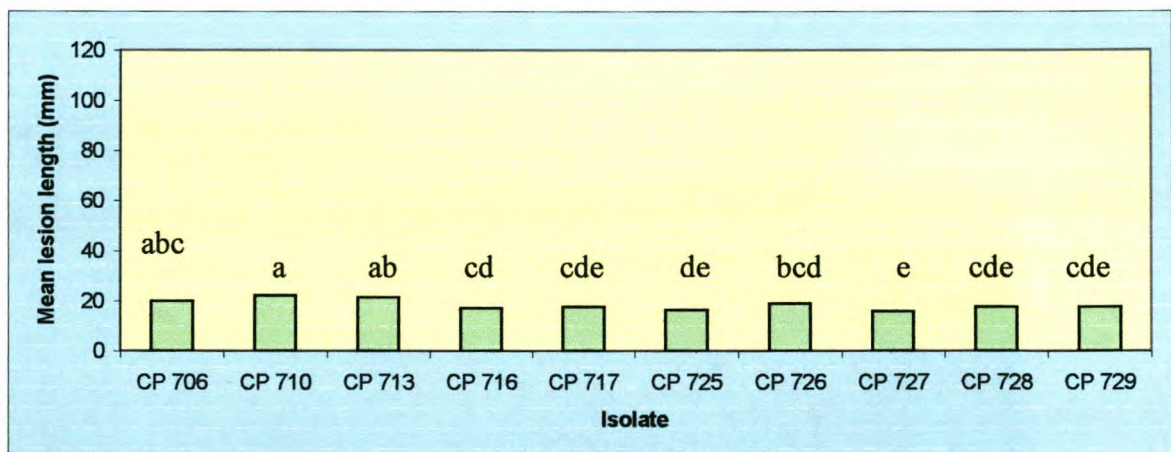


Figure 4 Aggressiveness of isolates expressed as mean lesion length. Means were calculated on the combined data of five rootstocks. Each bar represents means of ten lesion lengths measured after inoculation in May-99. Bars topped by the same letter do not differ significantly ( $P \leq 0.05$ ).

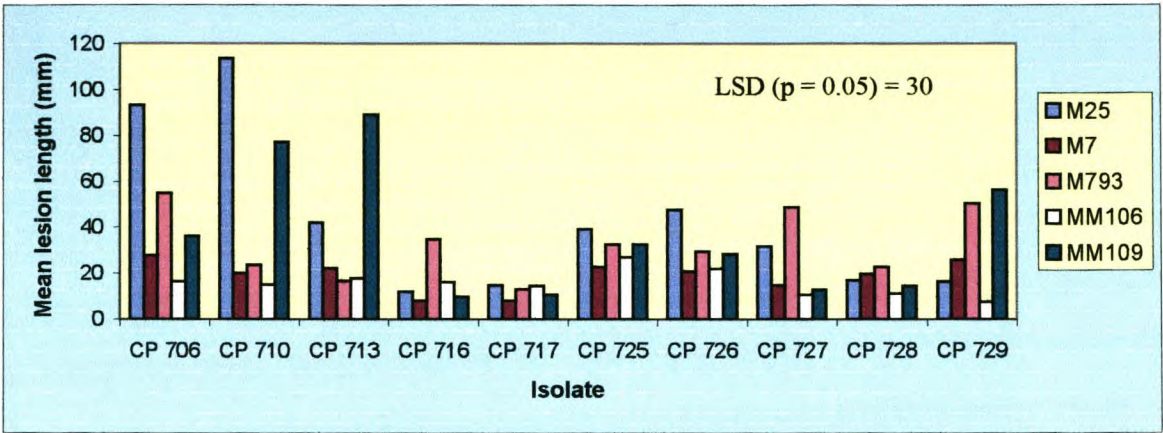


Figure 5 The aggressiveness of isolates as expressed by mean lesion length on five rootstocks. Bars represent means of three lesion lengths measured in February-99.

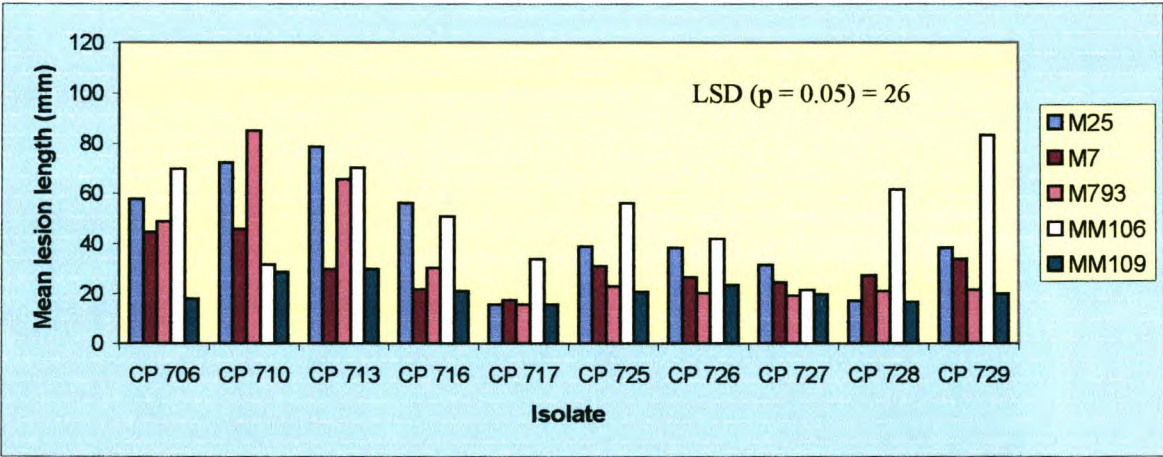


Figure 6 The aggressiveness of isolates as expressed by mean lesion length on five rootstocks. Bars represent means of six lesion lengths measured in March-99.



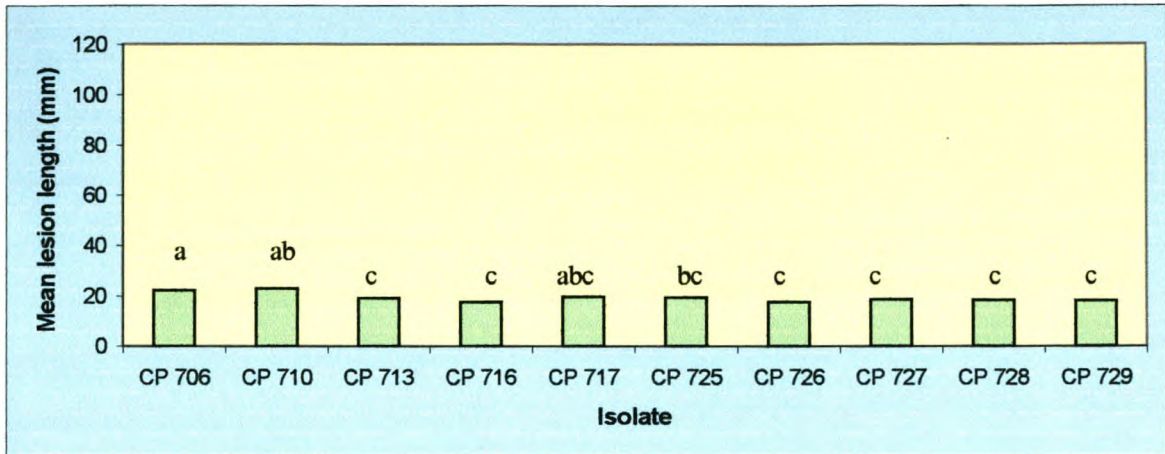


Figure 7 Aggressiveness of isolates expressed as mean lesion length. Means were calculated on the combined data of five rootstocks. Each bar represents a mean of 25 lesion lengths measured from three inoculation dates during year 2000. Bars topped by the same letter do not differ significantly ( $P \leq 0.05$ ).

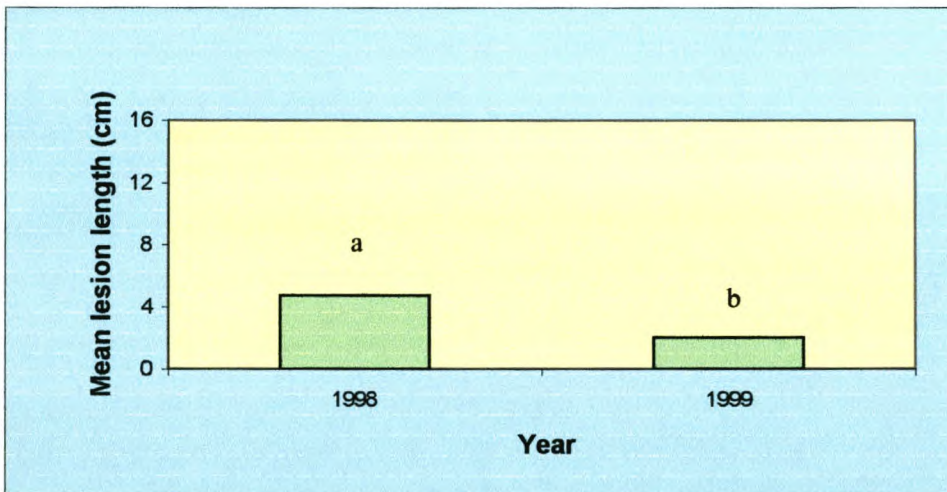


Figure 8 The effect of year of inoculation expressed on lesion development. Each bar represents a mean of 150 lesion lengths measured on the intact stems after 2 months of incubation in the greenhouse. Bars topped by the same letter do not differ significantly ( $P \leq 0.05$ ).

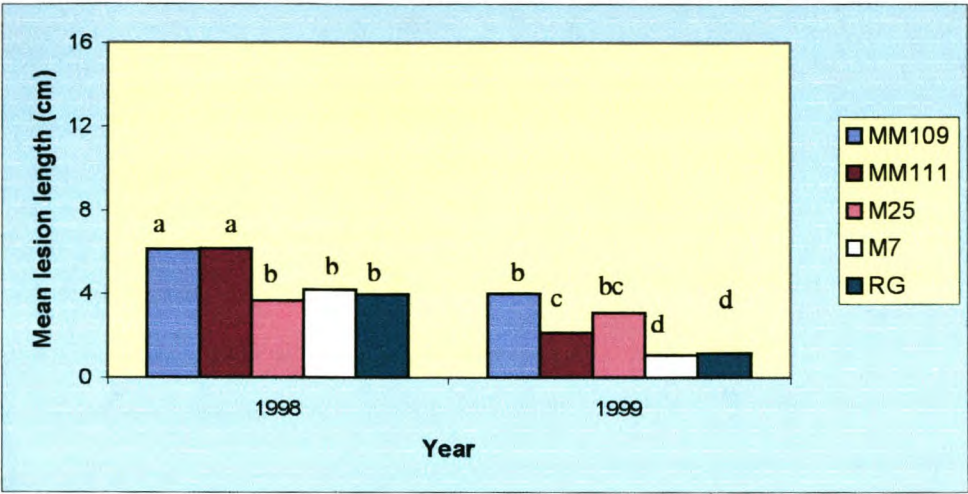


Figure 9 Rootstock susceptibility expressed as mean lesion in 1998 and 1999. Each bar represents a mean of thirty lesion lengths measured on the xylem tissue after 2 months of incubation in the greenhouse. Bars topped by the same letter do not differ significantly ( $P \leq 0.05$ ).

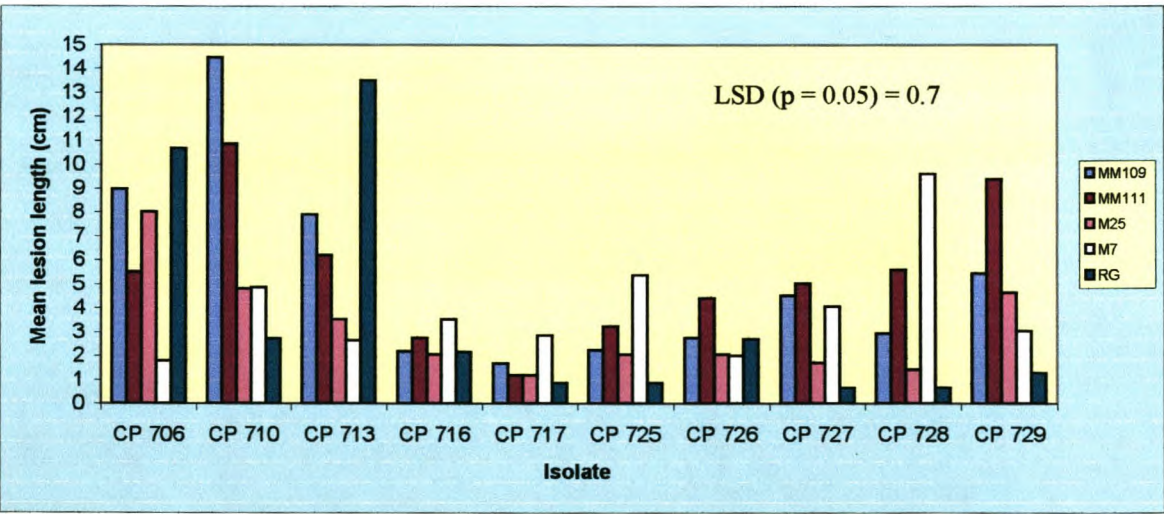


Figure 10 The aggressiveness of isolates as expressed by mean lesion length on five rootstocks. Means represent three lesion lengths measured 2 months after inoculation of intact stems with *Phytophthora cactorum* mycelium.



### 3. Use of an *in vitro* excised shoot bioassay to evaluate seasonal effects on apple rootstock resistance against *Phytophthora cactorum*

#### Abstract

Shoots of five apple rootstocks, MM106 (susceptible), MM109 (moderately susceptible), M7 (moderately resistant), M793 (moderately resistant), and M25 (susceptible) were inoculated with a single *Phytophthora cactorum* isolate once a month for 26 months (March-98 through April-00) using an *in vitro* excised shoot bioassay. Rootstock susceptibility was measured as lesion length after 14 days of incubation at 25°C in the dark. Rootstock susceptibility was low during dormancy in winter and high during active growth in summer, except for MM106, since its susceptibility pattern occurred 1-2 months later. Seasonal patterns of rootstock susceptibility to infection were similar in both years. Rootstock susceptibility depended on physiological growth stage, thus a proper choice of inoculation intervals is vital in determining susceptibility of rootstocks to infection.

#### Introduction

Crown rot caused by *Phytophthora cactorum* (Lebert & Cohn) Schröt. is an important disease on apple trees worldwide and has recently been recognized as being economically important in South Africa (Van der Merwe and Mathee 1973). *Phytophthora* associated diseases in apple orchards have been traditionally controlled by application of systemic fungicides such as metalaxyl and fosetyl-Al. Difficulty in predicting disease outbreak complicates economic management by chemical treatments and the emergence of strains of the pathogen resistant to chemicals has limited this disease control option. Of the various options available to manage this disease, the use of resistant rootstocks with desirable horticultural properties and genetic resistance to the pathogen is both environmentally considerate and economically appealing (Browne and Mircetich 1993).

A number of techniques have been used to test rootstock resistance in various apple-growing regions of the world (Borecki and Millikan 1969; Sewell and Wilson 1973; Bielenin



1977; Dakwa and Sewell 1981; Jeffers and Aldwinckle 1986; Utkhede and Quamme 1988; Browne *et al.* 1993). Many variables can affect results of such bioassays. Choice of inoculation date is an important variable that can affect the susceptibility of trees to infection. Susceptibility of apple rootstocks to *Phytophthora* spp. is known to fluctuate seasonally (Bielenin 1977; Jeffers *et al.* 1981; Browne *et al.* 1990; Browne and Mircetich 1996) and point sampling does not give reliable separation of resistance properties (Utkhede *et al.* 1988) because it only looks at one point thus ignoring the variation which occurs over the seasons.

Season affects lesion development (the measure of susceptibility) in a number of ways. The aggressiveness of isolates, or conversely, the relative susceptibility of host plants, is affected by season. For example Bielenin (1977) did not observe differences among rootstocks during dormancy, rather the best separation of rootstock susceptibility occurred during flowering (spring to early summer). On the other hand, Utkhede *et al.* (1988) found the best separation of resistance properties if testing commenced when rootstocks were dormant. Best separation of isolate aggressiveness occurred when rootstocks were dormant (Part 2), but season by rootstock interaction prevented direct comparison of rootstocks.

The effect of month of inoculation on the severity of disease also depends upon which species of *Phytophthora* was used. Jeffers *et al.* (1986) used an *in vitro* assay to study the seasonal variation in the colonization of two apple rootstocks (MM111 and MM106) inoculated with five *Phytophthora* spp. They observed that *P. cactorum* and *P. cambivora* Petri (Buisman) only had one peak period when rootstock colonization was the highest, during late spring and summer, whereas *P. cryptogea* Pethybr. & Laff., *P. megasperma* Drehsler and an unidentified *Phytophthora* spp. had two peaks, one during summer and the second one during winter. Browne *et al.* (1996) also found that rootstocks were most susceptible to *P. cactorum* and *P. cambivora* in summer whereas rootstocks were most susceptible to *P. cryptogea* only during the winter.

There are a number of variables that can influence the results and interpretation of a screening test, thus it is important to reduce the number of variables so that the results can be sensibly interpreted. Use of a representative isolate of the pathogen is desirable but until issues regarding the host specificity of *P. cactorum* are resolved (Part 2) the isolate effect has



to be simplified either by using a single isolate or a mixture of isolates. Using a single isolate of the pathogen can best achieve this and most resistance tests reported have only used a single isolate of the pathogen (Sewell *et al.* 1973; Bielenin 1977; Dakwa *et al.* 1981; Jeffers *et al.* 1986; Utkhede *et al.* 1988; Browne *et al.* 1993).

Since point sampling is not an acceptable method to employ in resistance testing, it is important to identify and quantify the extent and constancy of fluctuations in host resistance over time. Furthermore, information on the epidemiology of the disease can be acquired, which can then be implemented in integrated disease control. The aims of this research were to (1) determine seasonal variations in the susceptibility of five apple rootstocks in a period of 26 months, and (2) compare periods of highest colonization by *P. cactorum* in South Africa with those reported elsewhere.

## Methods

**Collection of rootstock material** Shoots were collected from five apple rootstocks of known resistance, MM106 (susceptible, Jeffers *et al.* 1986), MM109 (moderately susceptible, Sewell and Wilson 1959), M7 (moderately resistant, Utkhede and Smith 1993), M793 (moderately resistant, Sewell *et al.* 1959), and M25 (susceptible, Utkhede *et al.* 1988). Collection of rootstocks was done at monthly intervals for 26 months, from March 1998 through April 2000. From March 1998 through to December 1998 rootstock shoots were collected from an open air local nursery and as from January 1999 until April 2000 shoot collection was from rootstocks growing under 40% black shade net. Both the nursery- and shaded rootstocks were subjected to similar cultural management, i.e. irrigation and fertilization. Shoots collected between September and December in each year were from the previous growing season's shoots because current season's shoots were too young and succulent for inoculation.

**Inoculum** A single South African *P. cactorum* isolate obtained from the crown of a diseased apple tree (Pink Lady/MM106) (Part 2) was used for monthly inoculations. The isolate was stored in McCartney bottles with sterile distilled water at 25°C (Boesewinkel 1976). A week prior to inoculation the isolate was plated on PDA.

Monthly inoculations were performed using an excised shoot assay of Jeffers *et al.* (1981). Eighty milliliters of PDA plus 20ml/L benomyl (0.1g in 100 ml sterile distilled water)



and 1 ml/L rifampicin (0.025g in 1ml 5% DMSO) were dispensed into glass jars. Jars were inoculated with a 4-mm PDA plug bearing *P. cactorum* mycelium and incubated in the dark at 25°C until mycelia growth covered the entire agar surface. Control jars were inoculated with sterile PDA plugs.

Seventy millimeter-long shoot segments were cut from the central portions of growing shoots. The shoot segments were surface sterilized in 70% alcohol and in 1% commercial sodium hypochloride solution (with 3ml Tween) for 10 min respectively. Shoots were then rinsed three times with sterile distilled water. The basal end of each shoot was cut on opposite sides (excision) with a sterile scalpel to expose the phloem-cambium tissue. Five (i.e. one of each rootstock type) excised shoots were inserted vertically, distal ends up into *P. cactorum* mycelia growth (Figure 1). Thirty jars were inoculated each month. Jars were sealed with parafilm and incubated for 14 days in the dark at 25°C. After incubation, lesion and excision lengths on each shoot were measured and the actual lesion length calculated from the difference between measured lengths.

**Statistical analysis** Data were analyzed by regression analysis procedures (SAS Institute, Cary, N.C.). Mean lesion lengths (at 95% confidence levels) were plotted over time to produce seasonal patterns of the extent of colonization of five rootstocks (MM106, MM109, M7, M793 and M25) by a single *P. cactorum* isolate.

## Results

No lesions developed on the control rootstocks that were inoculated with PDA discs only. Direct inoculations onto stems of apple rootstocks revealed marked seasonal differences in susceptibility to the crown rot pathogen, *P. cactorum* (Figure 2). All inoculated rootstocks showed cyclical variation in lesion length over the different seasons of the year. The pattern of lesion development was very consistent over the 26-month period (Figure 2). In general maximum lesion development occurred during the summer months (November to February) while minimum lesion development occurred in winter (June to September). There was a significant shift in phase of the cyclical pattern of MM106 where it was about two months out of phase with the other rootstocks tested (Figure 2). Thus colonization of four rootstocks (MM109, M7, M793 and M25) increased to a maximum in summer (December) and decreased



to a minimum in winter (June / July). Whereas in rootstock MM106 maximum lesion length was recorded in February and minimum lesion length in August / September.

## Discussion

Data presented confirm previous reports in the seasonal variation of rootstock susceptibility (Bielenin 1977; Jeffers *et al.* 1986; Utkhede *et al.* 1988; Browne *et al.* 1990; Browne *et al.* 1995). In summer the disease organism overwhelms possible resistance of rootstocks in new, actively growing shoots. The reason for this increased susceptibility of rootstocks during active growth is not clearly understood, but may be related to active growth and shoot succulence. Many factors that govern seasonal changes in susceptibility to *P. cactorum* remain unknown. This is because direct inoculation bypasses events that precede and accompany natural host penetration (Jeffers *et al.* 1986; Browne *et al.* 1996). The role of environmental factors prior to testing and how they may have affected the host response is not known and was not investigated during this study.

These results have revealed new information regarding changes in the relative resistance of the different rootstocks over the growing season. Thus from February through June MM106 appears to be the most susceptible rootstock which is concordant with previous reports (Jeffers *et al.* 1986), but from June to January it performs as the most resistant rootstock. In separate tests (Part 2) this “cross over” of resistance was also found. This finding has important implications in deciding on the way to interpret resistance test results, and emphasizes the importance of not relying on point sampling. Furthermore, useful information has been acquired regarding the epidemiology of the disease with regard to “windows of susceptibility”. The practical application of this information can be extended to timely applications of chemical or other prophylactic treatments, which also makes disease management with these agents economically attractive. The phenomenon of phase shift in susceptibility of different rootstocks needs to be tested on a broader scale to assess whether it has any practical application to refinement of the resistance testing technique and interpretation of the results.



## References

- Bielenin, A. (1977) - Collar rot of apple trees. II . Seasonal fluctuations in susceptibility of apple trees to *Phytophthora cactorum* (Leb. et Cohn.) Schroet. *Fruit Science Reporter* **4**: 27-39.
- Boesewinkel, H.J. (1976) - Storage of fungal cultures in water. *Transactions of the British Mycological Society* **66**: 183-185.
- Borecki, Z. and Millikan, D.F. (1969) - A rapid method for determining the pathogenicity and factors associated with pathogenicity of *Phytophthora cactorum*. *Phytopathology* **59**: 245-247.
- Browne, G.T. and Mircetich, S.M. (1993) - Relative resistance of thirteen apple rootstocks to three species of *Phytophthora*. *Phytopathology* **83**: 744-749.
- Browne, G.T. and Mircetich, S.M. (1996). Effects of month of inoculation on severity of disease caused by *Phytophthora* spp. in apple root crowns and excised shoots. *Phytopathology* **86**: 290-294.
- Browne, G.T. Mircetich, S.M. and Willamowski, H.D. (1990) - Seasonal variation in susceptibility of apple rootstock to three *Phytophthora* spp. in excised shoots and intact stems. (Abstr.) *Phytopathology* **80**: 887.
- Dakwa, J.T. and Sewell, G.W.F. (1981) - Influence of rootstock type and time of inoculation on the resistance of five apple scion cultivars to collar rot caused by *Phytophthora cactorum*. *Journal of Horticultural Science* **56**: 357-362.
- Jeffers, S.N. and Aldwinckle, H.S. (1986) - Seasonal variation in extent of colonization of two apple rootstocks by five species of *Phytophthora*. *Plant Disease* **70**: 941-945.
- Jeffers, S.N., Aldwinckle, H.S., Burr, T.J. and Arneson, P.A. (1981) - Excised twig assay for the study of apple tree crown rot pathogens in vitro. *Plant Disease* **65**: 823-825.
- Sewell, G.W.F. and Wilson, J.F. (1959) - Resistance trials of some apple rootstock varieties to *Phytophthora cactorum* (L. & C.) Schroeter. *Journal of Horticultural Science* **34**: 51-58.
- Sewell, G.W.F. and Wilson, J.F. (1973) - *Phytophthora* collar rot of apple: Seasonal effects on infection and disease development. *Annals of Applied Biology* **74**: 149-158.



- Utkhede, R.S. and Quamme, H.A. (1988) - Use of excised shoot assay to evaluate resistance to *Phytophthora cactorum* of apple rootstock cultivars. *Canadian Journal of Plant Science* **68**: 851-857.
- Utkhede, R.S. and Smith, E.M. (1993) - Long-term effects of chemical and biological treatments on crown and root rot of apple trees caused by *Phytophthora cactorum*. *Soil Biology and Biochemistry* **25**: 383-386.
- Van Der Merwe, J.J.H. and Mathee, F.N. (1973) - *Phytophthora* crown and root rot of apple trees in South Africa. *Phytophylactica* **5**: 55-58.



Figure 1 Five apple rootstock shoots inserted in mycelia growth of *Phytophthora cactorum*.



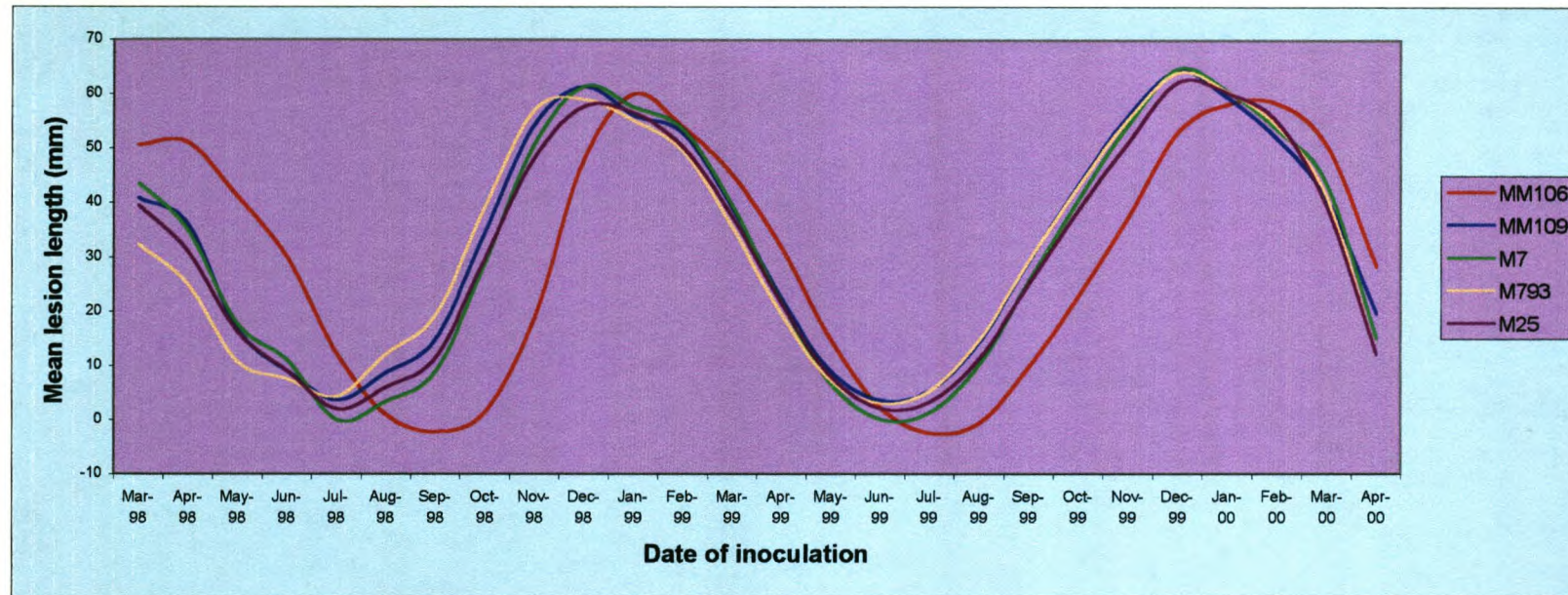


Figure 2 Seasonal variation in susceptibility of excised shoots of five apple rootstocks inoculated with *Phytophthora cactorum* *in vitro*. Thirty shoots of each rootstock type were inoculated monthly and lesion lengths measured after 14 days of incubation in the dark at 25°C.



## 4. Evaluation of two inoculation techniques for the assessment of resistance of apple seedlings against *Phytophthora cactorum*

### Abstract

Two inoculation techniques were evaluated in assessing apple seedling resistance to *Phytophthora cactorum*. With the sand-bran inoculation technique, inoculum was cultured on a sterile river sand with digestive wheat bran medium. It was mixed with sterile planting medium to make up different concentrations of mass inoculum to mass of planting medium. Seedlings were then transplanted into inoculated soil and disease was rated after 13 weeks. In the second technique, zoospore suspensions were used as inoculum and suspensions of three different concentrations of zoospores were used. Zoospore release was induced on 14-day-old mycelium grown on V-8 plates. Disease was rated after 13 weeks. Seedling dry root weight was used as a measure of aggressiveness of isolates, effectiveness of concentrations and susceptibility of seedlings of different age groups. All tested isolates were pathogenic but differed significantly in their levels of aggressiveness. At lower inoculum concentrations there were no significant differences in disease levels but at higher concentrations significantly lower dry root weights were recorded. Two-, three- and four-month-old seedlings were significantly more susceptible to disease than were 5-month-old seedlings.

### Introduction

*Phytophthora cactorum* (Lebert & Cohn) Schröt. occurs worldwide and is responsible for major losses in apple production where it causes collar, crown and root rot in apple trees (Parker 1979; Erwin and Ribeiro 1996). This pathogen can also cause seedling diseases. The first visible symptoms of infection on seedlings are light green discoloration of leaves or purple-red coloring of leaves (Van der Merwe and Mathee 1973) as illustrated in Figure 1. Chemical control is not suitable in the long term because it is expensive and pathogens develop resistance to the most effective chemicals which are systemic and with the emphasis on high standards of food safety, alternative methods for disease management must be sought. The use of cultivars and rootstocks that are disease resistant is economical and environmentally considerate.



Apple trees become susceptible to crown rot as they mature (McIntosh 1968; Jeffers and Wilcox 1990; Harris 1991). McIntosh (1968) suggested that seedlings that can not survive inoculation with *P. cactorum* would probably not survive disease as mature trees. Conversely, seedlings that do survive inoculation might not necessarily survive an attack by the pathogen when they are mature and survivors must therefore endure stringent testing under a range of different conditions before a genotype can be designated as resistant. Thus, screening trials are the first phase of eliminating highly susceptible seedlings (pre-screening).

Different researchers have employed a variety of techniques in pre-screening trials (McIntosh 1968; Watkins and Werts 1971; Aldwinckle *et al.* 1975; Browne and Mircetich 1993). An ideal technique should allow natural infection at the most susceptible stage of tree development with an appropriate concentration of inoculum under disease-conducive conditions (temperature and humidity). Browne *et al.* (1993) used vermiculite media as inoculum by mixing it with planting media. Three months after transplanting they assessed resistance by measuring crown rot length, rating crown rot girdling and percentage root rot. They concluded that expressions of resistance to *P. cactorum* in apple rootstocks did not consistently extend to *P. cambivora* and *P. cryptogea*, also that relative resistance of different apple rootstocks varied with species of *Phytophthora* used. Watkins *et al.* (1971) compared the susceptibility of five seedling families to *P. cactorum*. They concluded that zoospore inoculations followed by stem inoculations might provide an efficient two-step procedure for selecting resistant plants. McIntosh (1968) inoculated 2 to 3-week-old seedlings by flooding with zoospore suspensions and used seedling survival as a measure of resistance. Aldwinckle *et al.* (1975) inoculated open pollinated McIntosh seedlings with zoospore suspension. Seedlings that survived after two weeks were considered resistant.

In spite of all the work done, there is no consensus on which technique to apply for seedling selections. There is a lack of standardization of procedures regarding (a) type or form of inoculum used, (b) concentration of inoculum, (c) age of seedlings, (d) temperature at which the trials should be conducted, (e) duration of trials, (f) planting medium, (g) irrigation regimes (flooding or not) and (h) resistance rating. The aim of this study was to investigate the effects of some of the above mentioned variables on disease development so that pre-screening techniques suitable for selection of seedlings can be optimized.



## Methods

**Inoculum** Five *P. cactorum* isolates (Table 1) obtained from four localities in the Western Cape province were used. Isolates were stored in McCartney bottles with sterile distilled water at 25°C. Seven days before inoculation, isolates were individually transferred onto potato dextrose agar (PDA) plates. Sand-bran inoculum (Lamprecht 1986) was prepared by mixing and autoclaving 400 g of washed river sand with 20 g wheat bran and 60 ml distilled water in 500 ml Schott bottles (Figure 2). Ten, 4-mm PDA plugs bearing *P. cactorum* mycelium were used to inoculate the sterile sand-bran mixture (one isolate per bottle). Additionally, a combination of all five isolates (two agar plugs per isolate) and a control (PDA plugs only) were also used. Thus, there was a total of seven isolates. The inoculum was incubated for 14 days in the dark at 25°C. To ensure even growth of mycelia throughout the medium the bottles were shaken by hand every second day.

Zoospore inoculum was prepared by growing *P. cactorum* isolates on PDA for two weeks. The cultures were then cut into small square pieces and flooded with 10-ml sterile distilled water per plate to enhance sporangia production. The water was poured off 30-min later and plates were re-incubated to allow sporangia to develop. After 3-5 days, plates were flooded and incubated at 4°C for two hours to induce the production and release of zoospores. Zoospore release was induced separately for each isolate. The resultant zoospore suspensions were amended with sterile water to attain required concentrations ( $2 \times 10^4$ ,  $3 \times 10^4$  and  $4 \times 10^4$  zoospores per ml).

## Evaluation of resistance using a sand-bran inoculation technique

**Jonathan seedlings** The aggressiveness of isolates and effects of inoculum concentration on disease were assessed using cv. Jonathan seedlings. Jonathan seeds were stratified for 3 months and germinated in perlite medium. After two weeks in perlite medium seedlings were transplanted into sand:bark (1:1) soil mixture for 13 weeks. After 13 weeks seedlings were transplanted into sterile sand-bark planting medium inoculated at concentrations of 0.0, 0.5, 1.0, 2.0, 5.0 and 10.0% (mass of sand-bran inoculum to mass of planting medium). The trial was arranged in a complete randomized block design. Inoculated seedlings were kept in a greenhouse at 25°C and were watered twice a day (morning and afternoon) with municipal



water for 13 weeks. This trial commenced on (13/10/98) and was repeated (04/11/99). Dry root weight was recorded as a measure of isolate aggressiveness and disease severity.

*Braeburn seedlings* The effect of inoculum concentration on disease severity was tested on 13-week-old cv. Braeburn seedlings. Braeburn seedlings were transplanted into soil inoculated with one highly aggressive isolate at concentrations of 0.0, 1.0, 5.0, 25.0 and 50.0% using the sand-bran technique. Seedlings were kept at 25°C in a greenhouse and watered as described before for 4 weeks. This trial commenced on (13/10/98) and was repeated on (11/06/99) and on (19/05/00). The trial was arranged in a complete randomized block design. Dry root weight was recorded as a measure of isolate aggressiveness and disease severity.

*M25 clonal material* The influence of seedling age on the susceptibility of M25 clones was tested using one highly aggressive isolate. Seedlings of ages, 2, 3, 4 and 5-months-old were transplanted into soil inoculated with sand-bran inoculum at 0.0, 1.0, 5.0, 10.0 and 25.0% concentration levels. The trial was arranged in a complete randomized block design, and kept at 25°C in a greenhouse. Seedlings were watered as described before for 8 weeks. This trial commenced on (30/11/99) and was terminated on (14/02/00). Dry root weight was recorded as a measure of disease severity.

### **Evaluation of resistance using a zoospore inoculation technique**

*Braeburn seedlings* Seven-week-old cv. Braeburn seedlings grown in a sterile sand:bark (1:1) planting medium were inoculated with 5 isolates (Table 1). One milliliter aliquots of zoospore suspension of each isolate at concentrations of  $2 \times 10^4$ ,  $3 \times 10^4$  and  $4 \times 10^4$  zoospores per ml were used to inoculate seedlings. Seedlings were inoculated by removing soil to expose the roots and 1-ml aliquot was then dispensed onto the roots. Plants were then replanted in sterile sand-bark medium and watered lightly. Eighteen seedlings were inoculated with one isolate at each concentration per treatment. Control seedlings were inoculated with 1-ml aliquot of sterile distilled water. Two treatments were included, drench and non-drench. Seedling trays were placed in plastic tubs and watered until flooding occurred. Trays were left standing in water for 48 hours. Seedlings were drenched one week after inoculation and thereafter once a week. Non-drench seedlings were watered twice a day (morning and afternoon). Seedlings



were kept in the greenhouse for 8 weeks at 25°C. This trial commenced on (13/05/98) and was repeated on (26/05/99). The trial was arranged in a complete randomized block design. Dry root weight was recorded as a measure of isolate aggressiveness and disease severity.

**Statistical analysis** Mean dry root weight was calculated for the various treatments. Data were analyzed for significant differences among isolates, concentrations, seedling age and treatment. Levene's test for heterogeneity of variance (Snedecor and Cochran 1980) was carried out on all data. Data were also tested for a lack of normality using variance test for normality (Shapiro and Wilk 1965). There was no significant heterogeneity and data was normally distributed. Analysis of Variance was carried out and Student's t-LSD tests were performed in the case of significant effects.

## **Results**

### **Evaluation of resistance using a sand-bran inoculation technique**

*Jonathan seedlings* Significant year x isolate interaction occurred, therefore data for each year were analyzed separately (Table 2). In the separate analysis significant differences were detected among the isolates ( $P = 0.0001$ ) and ( $P = 0.0299$ ) in 1998 and 1999, respectively (Table 2). In both years all the isolates significantly reduced the dry root weight (Figure 3) and thus were pathogenic to apple seedlings. In general the isolates were more aggressive in 1998 and caused seedlings to have lower root weight than in 1999. In 1998 isolate CP 725 was significantly less aggressive than other isolates (Figure 3). The mixture of isolates was more aggressive than CP725, less aggressive than isolate CP658 but not different from CP 711, CP 713 or CP 766 (Figure 3). Even though isolate CP 658 was the most aggressive, it was not significantly different from CP 711, CP 713 and CP 766. In 1999 there was a level of reduced aggressiveness, and differences among isolates were less pronounced. Furthermore, two of the most aggressive isolates in 1998, CP658 and CP713 fell into the least aggressive group in 1999. The mixture of isolates, CP 766 and CP 711 were more aggressive than CP658 and CP713, but did not differ from CP 725. In both years there were no significant differences between the inoculum concentrations tested.



*Braeburn seedlings* There was no significant year x concentration interaction thus main effects could be interpreted (Table 2). Significant differences among concentrations used to inoculate Braeburn seedlings were observed (Figure 4). There was no difference between the 1 % inoculum level and the control (0 %). The 5 % inoculum level was significantly less effective than 25 or 50 % in reducing root weight. The year effect was highly significant ( $P = 0.0001$ , Table 2). Results show that in 1998 the isolate used was significantly more aggressive than in 1999 which was not different to 2000 (Figure 5).

*M25 clonal material* Significant age x concentration interaction was observed (Table 2). There were no differences in the effects of inoculum concentration on four-month-old or younger seedlings, but significant differences in concentrations could be detected in five-month-old clones (Figure 6). Low mean root weight was obtained at 1 and 25 % levels of inoculum, while high mean root weight was recorded at 5 and 10 % inoculum. The control plants (0 % inoculum) had the highest mean root weight.

### **Evaluation of resistance using a zoospore inoculation technique**

*Braeburn seedlings* There was significant treatment x concentration interaction and treatment x isolate interaction (Table 3). This interaction prohibited sensible interpretation of the main effects and thus, separate analyses on the different treatments (drench or non-drench) were conducted. The only significant main effect in the non-drench treatment was the year effect where mean root weight recorded in 1999 was much larger than that recorded in 1998 (Figure 7). In the drench treatment the same year effect was noted but, additionally, there were significant differences among isolates (Table 3). The uninoculated plants had the highest root weight, even though seedlings inoculated with isolate CP 725 did not significantly differ from the controls (Figure 8). Isolate CP 713 was more aggressive on seedlings than any other isolate used. Isolate CP 725 was the least aggressive of all the isolates, with isolate CP 658, CP 711 and CP 766 being the intermediately aggressive isolates.

### **Discussion**

The significant decrease in the aggressiveness of isolates noted in many of the trials recorded here and previously (Part 2) from 1998 to 1999 can be attributed to the isolates' loss of



aggressiveness in culture. This emphasizes the importance of using freshly isolated strains of the pathogen for each test done. In some instances, there was interaction among isolates over two years and this can also be attributed to loss of aggressiveness, where on the first year isolates were highly aggressive but on the second year they became the least aggressive (Figure 3, isolate CP 658 and CP 713). Isolates differed in their aggressiveness in seedling trials as was found in stem bioassays and as previously reported (Bielenin 1977ab; Utkhede 1986; Harris 1991; Browne *et al.* 1993). The relative aggressiveness of isolates was similar irrespective of the testing technique. For example in the stem bioassays isolate CP 713 was highly aggressive in the stem bioassay as it was also in the sand-bran inoculation technique. Isolate CP 725 was the least aggressive in both techniques. This observation suggests that the inoculation technique did not influence the aggressiveness of isolates, although under different conditions, such as flooding, this might change. An intermediate level of aggressiveness was obtained using a mixture of isolates. It is easy and practical to apply a mixture of isolates using the sand-bran inoculation technique and this might be a feasible solution to the problem of using representative isolates to overcome the isolate rootstock interaction in seedling and potted plant trials.

At this stage no suitable inoculum concentration for the sand-bran technique can be recommended. Low concentrations were not effective in enabling decisive distinction of resistant genotypes. Although the 50% concentration level was effective this concentration is not recommended since the lower dry root weight could have resulted from a lack of water as half of the planting medium was sand and dried out quickly. The 25% inoculum level is recommendable, but this is still a high level of inoculum and would be impractical in large scale testing. The effect of flooding in combination with the sand-bran technique needs to be tested since this might allow use of a much reduced level of inoculum.

The duration of the test also needs to be finalized. In some tests dry root weight was weighed after 4 weeks and this might have been too soon to obtain a more definitive differentiation of resistant types. Browne *et al.* (1993) warned against placing too much emphasis on results evaluated after a short time. This comment is highly supported, but optimal test duration is yet to be attained. The criteria used by McIntosh (1968) provided



stringent evaluation criteria for assessing resistance and the sand-bran technique must be manipulated until the resistance classes outlined by McIntosh (1968) can be attained.

Age of seedlings used in tests emerged as an important factor, thus seedlings under five-months-old should not be used. Age of seedlings in combination with duration of trial will probably extend testing time to longer periods (a year or more). Thus, pre-screening is not as 'quick' a test as might be perceived. If older seedlings are used, larger containers will be required since seedlings will have larger root volumes. Thus testing beds should be constructed for such purposes.

The lack of significant differences among zoospore concentrations used makes it difficult to select an appropriate concentration for screening. However, results suggest that for effective inoculations of seedlings using zoospore as inoculum, higher concentrations should be used. This could be difficult to achieve since production of zoospores is a tedious and time-consuming technique. Furthermore, not all isolates have the same zoospore forming ability (S. Denman, University of Stellenbosch, personal observation) and this would complicate practical aspects of testing.

The reason that the drench treatment was more effective than the non-drench treatment was because when seedlings were flooded with water the activity, and number of zoospores were enhanced and under waterlogged conditions the roots of the seedlings were stressed by anaerobic conditions. Although zoospores are difficult to produce *in vitro*, they are considered the most common natural form of inoculum (Watkins *et al.* 1971; Browne *et al.* 1993) and this provides a further reason for testing the sand-bran inoculum technique under flood conditions.

## References

- Aldwinckle, H.S., Polach, F.J., Molin, W.T. and Pearson, R.C. (1975) - Pathogenicity of *Phytophthora cactorum* isolates from New York apple trees and other sources. *Phytopathology* **65**: 989-994.
- Bielenin, A. (1977a) - Collar rot of apple trees. I . Pathogenicity of isolates of *Phytophthora cactorum* (Leb. et Cohn.) Schroet. *Fruit Science Reporter* **4**: 15 - 26.
- Bielenin, A. (1977b) - Collar rot of apple trees. II . Seasonal fluctuations in susceptibility of apple trees to *Phytophthora cactorum* (Leb. et Cohn.) Schroet. *Fruit Science Reporter* **4**: 27-39.
- Browne, G.T. and Mircetich, S.M. (1993) - Relative resistance of thirteen apple rootstocks to three species of *Phytophthora*. *Phytopathology* **83**: 744-749.
- Erwin, D.C. and Ribeiro, O.K. (1996) - *Phytophthora* diseases worldwide. American Phytopathological Society Press: Minnesota.
- Harris, D.C. (1991) - The *Phytophthora* diseases of apple. *Journal of Horticultural Science* **66**: 513-544.
- Jeffers, S.N. and Wilcox, W.F. (1990) - *Phytophthora* crown, collar and root rots. In: *Compendium of apple and pear diseases* (Eds A.L. Jones and H.S. Aldwinckle). pp. 43-45. The American Phytopathological Society: Minnesota.
- Lamprecht, S. C. (1986) - A new disease of *Medicago truncatula* caused by *Cylindrocladium scoparium*. *Phytophylactica* **18**: 111-114.
- McIntosh, D.L. (1968) - Resistance to *Phytophthora cactorum* among open pollinated seedlings of apple varieties and species. *American Society for Horticultural Science* **93**: 71-76.
- Parker, K.G. (1979) - Common names of apple diseases. *Phytopathology news* **13**: 127-128. In: *Plant Breeding Reviews* **3**: 361-434 (Eds B.J. Parliman and G.A. White). AVI Publishing Company: United States of America.
- Shapiro, S.S. and Wilk, M.B. (1965) - An analysis of variance test for normality (complete samples). *Biometrika* **52**: 591-611.



- Snedecor, G.W. and Cochran, W.G. (1980) - Statistical Methods. Seventh Edition. The Iowa State University Press. pp. 253-254.
- Utkhede, R.S. (1986) - *In vitro* screening of the world germplasm collection for resistance to *Phytophthora cactorum* crown rot. *Scientia Horticulturae* **29**: 205-210.
- Van Der Merwe, J.J.H. and Mathee, F.N. (1973) - Phytophthora crown and root rot of apple trees in South Africa. *Phytophylactica* **5**: 55-58.
- Watkins, R. and Werts, J.M. (1971) - Pre-selection for *Phytophthora cactorum* (Leb. & Cohn) Schroet. resistance in apple seedlings. *Annals of Applied Biology* **67**: 153-156.

**Table 1 Host and origin of isolates used for the inoculation of apple seedlings**

Isolate name	Host of origin (Scion/Rootstock) <sup>A</sup>	Place of origin <sup>B</sup>
CP 658	Apple seedling	Stellenbosch
CP 711	Pink Lady/M25	Grabouw
CP 713	Pink Lady/MM106	Barrydale
CP 725	Selekta (strawberry)	Paarl
CP 766	Pink Lady	Stellenbosch

<sup>A</sup> - Selekta is a strawberry plant.

<sup>B</sup> – All isolates used were from the Western Cape province.



**Table 2 Analysis of variance of dry root weight data of cv. Jonathan seedlings inoculated with *Phytophthora cactorum* different concentration levels, cv. Braeburn seedlings inoculated with isolate CP 658 at five concentration levels, and M25 clonal seedlings of different ages inoculated with isolate CP 658 at five concentration levels**

Source of variation	Degrees of freedom	F ratio	P value
<b>Jonathan seedlings</b>			
<i>Combined Analysis</i>			
Year	1	136.33	0.0001
Year (Rep)	4	0.81	0.5205
Isolate	5	10.61	0.0001
Year x Isolate	5	7.42	0.0001
Concentration	4	1.24	0.2973
Year x Concentration	4	1.51	0.2045
Isolate x Concentration	20	1.08	0.3805
Year x Isolate x Conc.	20	0.88	0.6111
<i>October – 98</i>			
Rep	2	1.28	0.2850
Isolate	5	14.89	0.0001
Concentration	4	1.23	0.3068
Isolate x Concentration	20	0.85	0.6430
<i>November – 99</i>			
Rep	2	0.34	0.7124
Isolate	5	2.69	0.0299
Concentration	4	1.52	0.2095
Isolate x Concentration	20	1.11	0.3675
<b>Braeburn seedlings</b>			
Year	2	13.26	0.0001
Year (Rep)	9	1.40	0.2260
Concentration	4	22.25	0.0001
Year x Concentration	8	1.25	0.2986
<b>M25 clones</b>			
Rep	2	1.03	0.3673
Concentration	4	5.59	0.0012
Age	3	34.72	0.0001
Concentration x Age	12	2.63	0.0115

**Table 3 Analysis of variance of root dry weight of seven-week-old Braeburn seedlings inoculated with five *Phytophthora cactorum* isolates at different concentrations and subjected to drenching or no drenching treatment**

Source of variation	Degrees of freedom	F ratio	P value
<b>Combined Analysis</b>			
Year	1	431.32	0.0001
Year (Rep)	4	0.75	0.5588
Isolate	4	1.61	0.1764
Treatment	1	10.13	0.0019
Isolate x Treatment	4	4.25	0.0030
Concentration	2	0.11	0.8923
Isolate x Concentration	8	1.46	0.1800
Treatment x Concentration	2	3.15	0.0465
Isolate x Treatment x Concentration	8	0.79	0.6146
Year x Isolate	4	1.82	0.1292
Year x Treatment	1	0.24	0.6261
Year x Treatment x Isolate	4	0.33	0.8597
Year x Concentration	2	0.02	0.9831
Year x Isolate x Concentration	8	1.03	0.4192
Year x Treatment x Concentration	2	1.30	0.2757
Year x Isolate x Treatment x Conc.	8	1.01	0.4348
<b>Drench treatment</b>			
Year	1	215.62	0.0001
Year (Rep)	4	1.12	0.3544
Isolate	4	2.70	0.0396
Concentration	2	0.36	0.6985
Isolate x Concentration	8	0.94	0.4900
Year x Isolate	4	1.19	0.3259
Year x Concentration	2	0.52	0.5950
Year x Isolate x Concentration	8	1.02	0.4352
<b>Non – drench treatment</b>			
Year	1	207.10	0.0001
Year (Rep)	4	0.18	0.9461
Isolate	4	0.59	0.6726
Concentration	2	0.57	0.5680
Isolate x Concentration	8	1.00	0.4498
Year x Isolate	4	0.91	0.4646
Year x Concentration	2	0.78	0.4649
Year x Isolate x Concentration	8	0.98	0.4631





A



B

Figure 1 Leaf symptoms following infection of seedlings with *Phytophthora cactorum*.  
A - light-green discoloration, B - purple-red discoloration.



Figure 2 Sand-bran inoculum. The inoculum was kept at 25°C in the dark for 14 days before used in the inoculation of seedlings.

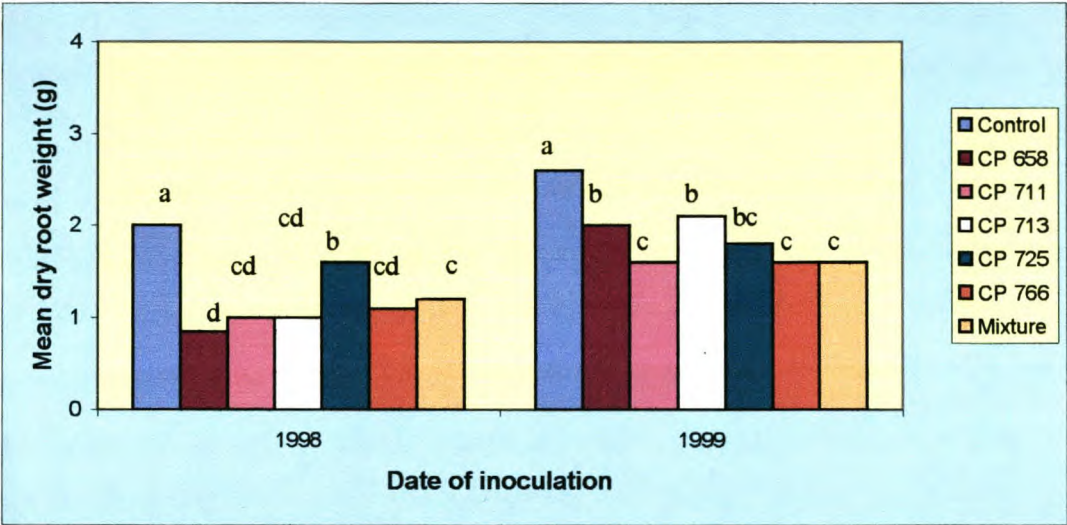


Figure 3. The aggressiveness of five *Phytophthora cactorum* isolates and a mixture of these isolates as expressed by mean dry root weight of cultivar Jonathan seedlings over a two-year period. Means are based on 90 seedlings inoculated per isolate for each trial. Bars topped by the same letter do not differ significantly ( $P \leq 0.05$ ).

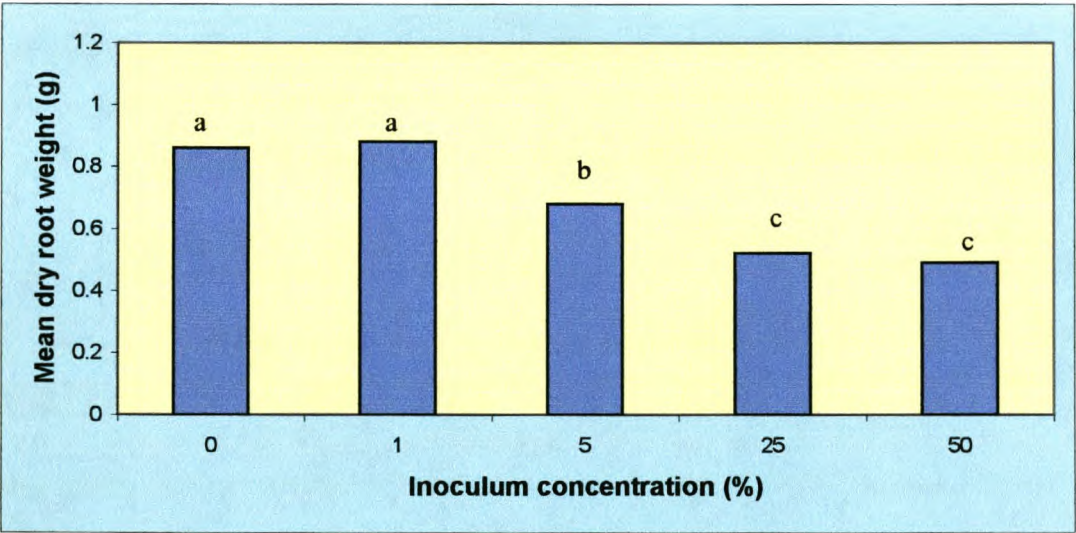


Figure 4. Disease severity expressed as mean dry root weight of Braeburn seedlings inoculated with a single, highly aggressive *Phytophthora cactorum* isolate at different inoculum concentrations. Means are based on 72 seedlings inoculated at each concentration. Bars topped by the same letter do not differ significantly ( $P \leq 0.05$ ).



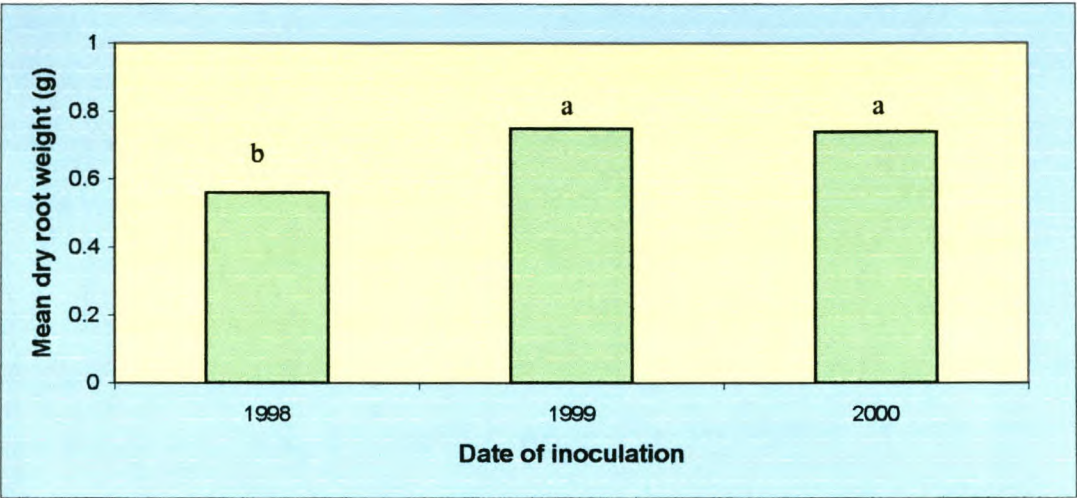


Figure 5. Disease severity expressed as mean dry root weight on 13-week-old Braeburn seedlings inoculated with a single highly aggressive *Phytophthora cactorum* isolate over three consecutive years. Means are based on 162 seedlings inoculated on each date. Bars topped by the same letter do not differ significantly ( $P \leq 0.05$ ).

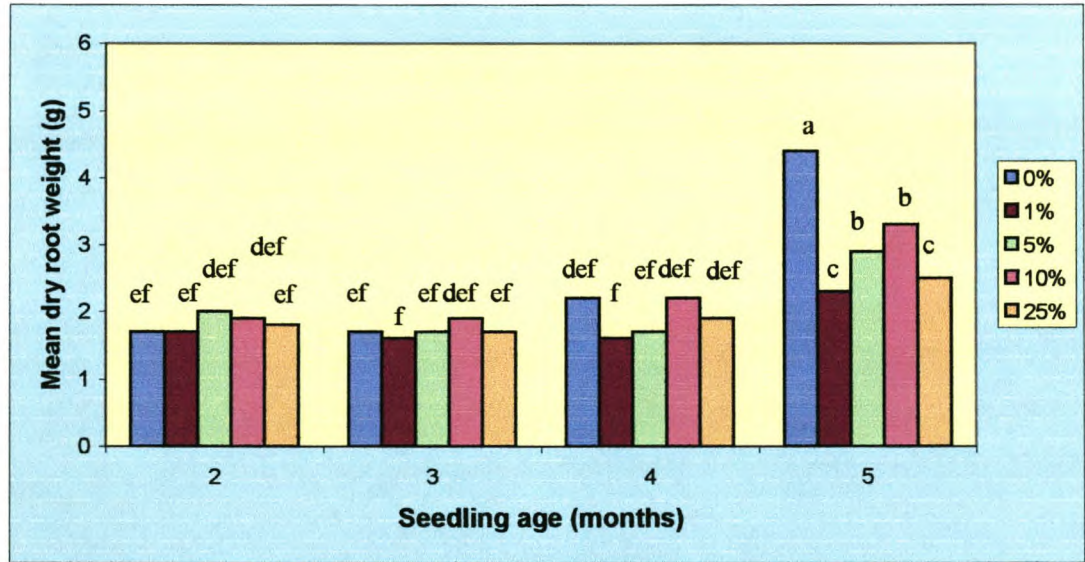


Figure 6 Disease severity as expressed by mean dry root weight on M25 clonal material of different ages inoculated with different concentrations. Each bar represent a mean of 18 inoculated clonal material at each concentration. Bars topped by the same letter do not significantly differ ( $P \leq 0.05$ ).

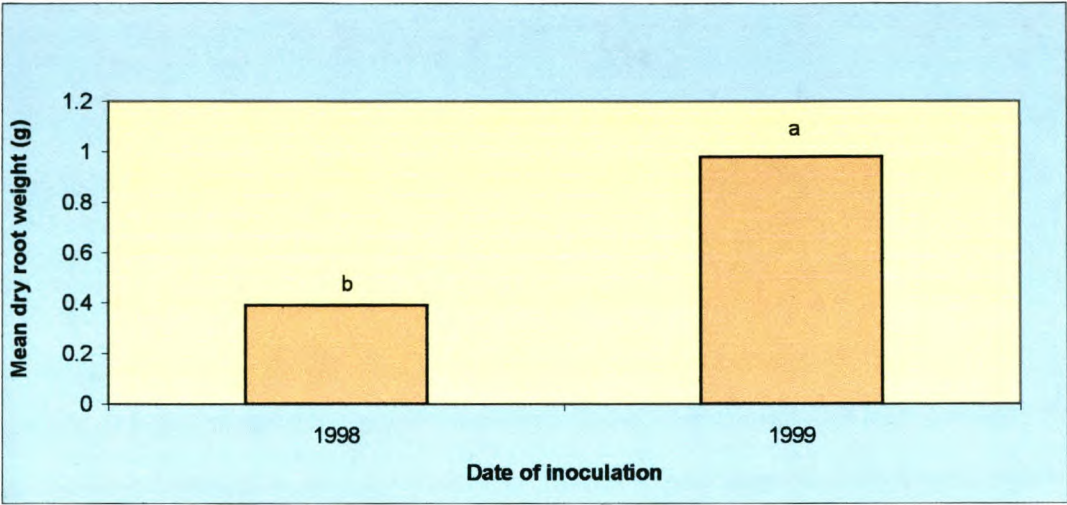


Figure 7 Disease severity expressed as mean dry root weight of Braeburn seedlings inoculated with zoospore inoculum over a two-year period. Each bar represents a mean of 590 inoculated seedlings. Bars topped by the same letter do not significantly differ ( $P \leq 0.05$ ).

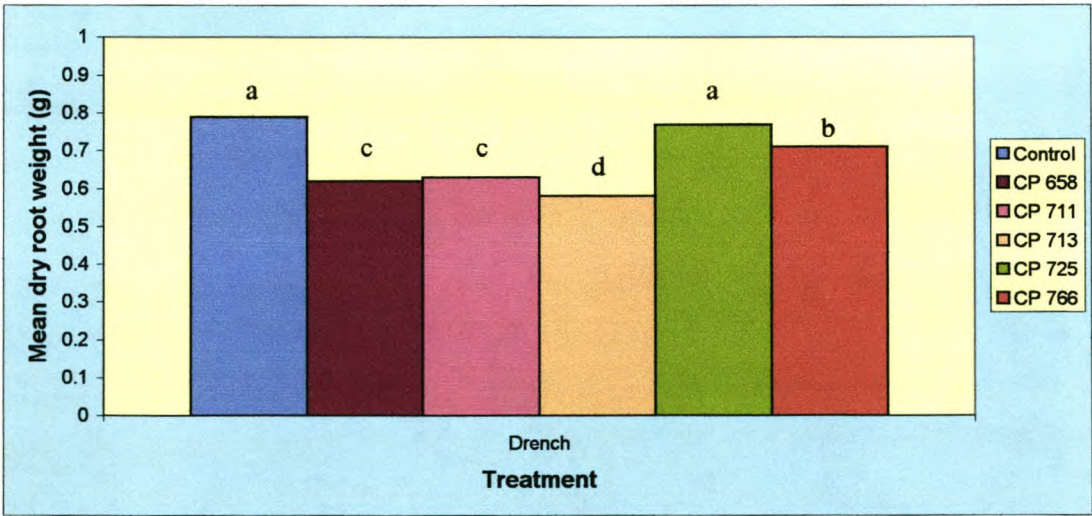


Figure 8. Aggressiveness of isolates as expressed by mean dry root weight of Braeburn seedlings inoculated with zoospores and flooded for 48-hours. Each bar represent a mean of 54 seedlings. Bars topped by the same letter do not differ significantly ( $P \leq 0.05$ ).